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FINAL REPORT OF A RESEARCH PROJECT
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Reporting Institution

National Institute for Research in Dairying,
Shinfield, Reading, Berkshire, England.

Principal Investigator

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Project Title

Isolation and characterization of selected enzymes of milk, to obtain fundamental information that will aid in improving the quality of dairy products.

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FOREWORD

The programme described in this report was carried out in the Physiology Department of the National Institute for Research in Dairying (head of department: Professor S.J. Folley). The principal investigator, Dr.H. Gutfreund, was given much help and support in the supervision of the programme by Dr. P.Andrews, a member of the permanent staff of the institute.

Miss L. Mendiola and Mr. E.M. Chance participated in the preliminary studies of enzyme assays of fractions from milk and mammary gland and Dr.T.E. Barman and Mr.W.K. Downey carried out the detailed studies on phosphatases and lipases respectively.

The provision of a preparative ultracentrifuge and counting equipment through a grant from the Rockefeller Foundation to the Physiology Department has made an essential contribution to these studies.

(I) INTRODUCTION

A complete solution of the problems posed for investigation in this project requires that one finds the answers to the following questions:

(1) What enzymes catalysing reactions of milk constituents occur in normal and abnormal milk?

(2) In what physical form do these enzymes occur; does storage or some abnormalities of the milk activate or inactivate them?

(3) What procedures should be used to prevent the activation of enzymes which catalyse reactions detrimental to milk or its constituents; if such activation occurs can it be reversed?

(4) What lessons can be learned about milk secretion from the pattern and physical state about enzymes in milk? In turn what physiological conditions would cause the secretion of enzymes detrimental to milk constituents?

During the initial phase of the programme a considerable amount of work was done to repeat classical studies on the distribution of enzymes in the different physical fractions of milk, the aqueous phase, the fat globules, the casein micelles and the nucleic acid (microsomal) particles. These general investigations were mainly helpful to us for gaining experience with the existing techniques for fractionating and assaying milk enzymes. They also helped us to decide on the problems on which it might be most profitable to concentrate our talents. It was subsequently decided to work mainly on two groups of enzymes, viz. the phosphatases and the lipases, and on their physical state in milk. Many of the problems touched upon in the early investigations on the distribution of enzyme protein in the different phases of milk were studied in detail with these particular enzymes, and are reported in the appropriate sections.

Progress in the study of enzymes and of the reactions they catalyse is very largely controlled by the availability of methods. The complexity of the reactions of isolated enzymes and the additional problems arising from attempts to study enzymes at concentrations and in the surroundings in which they occur, make demands for more sophisticated monitoring techniques. During the five years of this project a number of new techniques for the study of enzyme reactions were developed. Some of these techniques have found important applications in the study of milk enzymes, others are likely to do so in the near future.

This report is therefore devoted to three main topics: Techniques for the study of enzyme reactions; Phosphatases; and Lipases. Because the topic is of most direct and immediate practical concern the section dealing with lipases is the most detailed. A short section on milk esterase activity is also included.

(II) TECHNIQUES FOR THE STUDY OF ENZYME REACTIONS

(1) During the course of the investigations described in this report; as well as of others in progress in this laboratory, new techniques are required which can monitor the progress of

enzyme reactions with a continuous record of some physical quantity, which is proportional to the concentrations of the reactants. Continuous records of reactions give more objective data and greater latitude with the time scale of the experiments. However, one of the new techniques developed involves sampling reaction mixtures on a millisecond time scale for subsequent analysis.

There are two major areas in which new developments of techniques for following the time course of enzyme reactions are of importance. Firstly, the ability to measure very rapid reaction rates enables one to study enzymes over a wide range of concentration. It is often not appreciated that enzymes occur in physiological systems at very high concentrations and that they have different properties under these conditions as compared with the usual assay conditions. Also, as will be seen in the more detailed description of enzyme experiments, a great deal can be learned about the mechanism of enzymic catalysis, if one can get a record of the reaction during the initial few milliseconds while the first turnover of the enzyme-substrate reaction occurs. During this pre-steady state phase the individual steps of the reaction between enzyme and substrate can be detected (see enclosed reprint of paper on transients in enzyme reactions). Secondly it is of great value to be able to follow any one enzyme reaction by a variety of physical techniques such as optical density, fluorescence, calorimetry and pH changes. In a complex system, consisting either of several enzymes or of one enzyme reacting via a series of intermediates, the measurement of different physical changes occurring during the reaction gives the necessary information to identify the many parameters involved.

A wider range of procedures than that described for the present project is given by H. Gutfreund, An Introduction to the Study of Enzymes, Chapter IV; Wiley & Sons (1965).

(2) The recording of pH changes during the progress of enzyme reactions:

A large number of enzyme-catalysed reactions are accompanied by the liberation or withdrawal of hydrogen ions from the reaction mixture. It is relatively easy to make continuous observations of hydrogen ion concentrations either electrometrically or, in the presence of an indicator, spectrophotometrically. A variety of methods have been developed to follow enzyme reactions by means of some such principle.

Historically the first procedure for following pH changes continuously was developed for rapid reactions of carbonic anhydrase (Brinkman, Margaria & Roughton: Phil. Trans. Roy. Soc. A. 232, 65, 1933) and of proteolytic enzymes (Gutfreund: Disc. Faraday Soc. 20, 167, 1955). The spectrophotometric method was used for this purpose. More recently commercial equipment has been developed (Radiometer Company, Copenhagen) to give a continuous record of quantities of acid or alkali automatically added to the reaction mixture to keep the pH at a chosen constant value. This procedure provides automation and continuity in a procedure which has been used with manual additions and noting of time for much classical kinetic work on enzymic and non enzymic catalysis of hydrolysis reactions. Another procedure which has been used to some extent for biochemical reactions and which had the appeal of employing the manometric equipment usually available, involves the

use of carbonate buffer and the recording of the formation of CO_2.

The recent development of high impedance D.C. amplifiers with fairly rapid response times was, in the first place, largely due to their use in measuring the output from Geiger-Muller tubes. This equipment has also proved itself valuable for measuring small pH changes in conjunction with glass and calomel electrodes. As reported last year, full scale deflections on paper recorders can be obtained for 0.1 pH unit. For this purpose of following the course of rapid pH changes we have used an oscilloscope to record the output of the amplifier. This type of equipment allows one to measure the initial rate of the production of hydrogen ions without such change in pH as would affect the reaction in progress.

Well-characterised reactions such as the hydrolysis of amino acyl esters by proteolytic enzymes were used for the technical development of the above method. As a more severe test, enzyme systems involved in the phosphate metabolism of the cell were studied. During investigations of the pH-dependence of the hexokinase-catalysed phosphate transfer from adenosine-triphosphate (ATP) to glucose and of the reversible formation and hydrolysis of ATP catalysed by a mitochondrial enzyme system, the pH recording technique received its most critical evaluation.

The advantages of the direct electrometric recording technique are: (1) Simplicity of operation once it is set up; (2) Adaptability to measurements over a wide range of times from fractional seconds to hours; (3) If necessary the method can operate without stirring or additions during the period of the rate measurement.

The measurement of enzymic reactions over very short time periods has become of great interest both for the detection of rapidly formed reaction intermediates and for the study of enzymes under conditions similar to those appertaining in intracellular particleswhere local enzyme concentrations are often high and comparable with substrate concentration. We have found that with a relatively simple reaction vessel holding 3 ml. and being stirred with a magnetic stirrer the addition and homogeneous distribution of 0.2 ml. of enzyme to start the reaction can be easily achieved in 0.2 sec. For more rapid reactions more sophisticated mixing devices are being used but these are not being considered in connection with the present project.

(3) A rapid flow sampling technique:

During our studies of phosphatase it became apparent that it would be of considerable interest to develop a technique for taking samples from enzyme-substrate reaction mixtures, within milliseconds after initiation of the reaction. A variety of physical techniques make it possible to monitor a wide range of enzyme reactions from about 1 millisecond after mixing enzyme and substrate but many enzyme reactions, such as for instance the hydrolysis of phosphate esters or peptides, can not be followed without chemical analysis of time samples of the reaction mixture. We have, therefore, developed modifications of the Gibson stopped flow apparatus (Weissberger Investigation of Rates and Mechanisms of Reactions: 2nd Edition, 1963, vol. II, p. 742) to make it suitable for the collection of samples from approximately 3 milliseconds after mixing.

The first modification consisted of replacement of the hand-

driven barrier for pushing the syringes by a motor-driven one. The motor (torque 2lb-inches) is run continuously and the syringe drive is activated and stopped by a microswitch which controls the action of a magnetic clutch and a magnetic brake. When the microswitch is by-passed the brake goes off and the clutch engages and when an arm attached to the syringe drive touches an arm of the microswitch the clutch is switched off and the brake is switched on. The arm of the microswitch is adjustable so that the distance of syringe movement can be varied. This device was found to be suitable only to give an approximate measure of the volumes of reactants delivered from the two syringes. To determine these volumes accurately a millisecond timer (Electronic Instruments Ltd., Richmond, England; Chronotron Model 25E) was introduced into the circuit. This timer records the period for which the clutch is engaged and from this and the known rates of delivery at the two drive speeds used (7.0 ml./sec and 2.40 ml./sec of combined reaction mixture) the amounts of reaction mixture delivered can be calculated. Small magnets on the syringe barrels and the barrier keep the contact between them.

The second modification of the stopped-flow apparatus was the removal of the stopping syringe and the introduction of a clamp to mount capillary tubes (0.1 cm. diameter) of different lengths at a point 0.75 cm. past the mixing chamber. A moveable platform with a holder for a vessel (8 ml.) which contains the quenching solution can be adjusted to dip the end of the capillary, delivering the reaction mixture, into the quenching solution. The rate of movement of the reaction mixture from the mixing chamber through the capillary into the quenching solution is 0.87 cm/millisec. (at 7.0 ml./sec.) or 0.3 cm./millisec. (at 2.4 ml./sec.). The reaction time is determined by the length of the capillary tube.

For the experiments described here, during each operation approximately 3 ml reaction mixture was delivered into 3 ml of quenching solution. A suitable chemical reaction to test the performance of flow methods is the alkaline hydrolysis of 2:4 dinitrophenyl acetate. The rate of appearance of one of the products (dinitrophenol) can be followed either continuously in the stopped flow machine or after timed intervals in the quenching machine, by measuring the absorption at 360 mμ ($\varepsilon = 1.45 \times 10^4$/mole/cm. at pH 7 and 0.88×10^4 at pH 4.0). The 2:4 dinitrophenyl acetate concentration was kept constant in all the reaction mixtures at 0.625 m$\underline{\underline{M}}$, which means that the concentration in the syringe was 1.25 m$\underline{\underline{M}}$ in 5 m$\underline{\underline{M}}$ HCl. The syringe solutions were made up from 12.5 m$\underline{\underline{M}}$ dinitrophenyl acetate in isopropanol so that the final reaction mixture was 5% in isopropanol. The range of NaOH concentrations in the reaction mixture (0.237 $\underline{\underline{M}}$ to 0.47 $\underline{\underline{M}}$) resulted in pseudo first order constants for the hydrolysis of dinitrophenyl acetate. The samples taken at 7.1, 9.0, 13.0 and 17.5 msec. were obtained at a flow rate of 7 ml/sec, while the others were taken at a flow rate of 2.43 ml/sec. The reactions were quenched in 3 ml 4 $\underline{\underline{N}}$ HCl and immediately adjusted to pH 4 by the addition of 3 ml 10 $\underline{\underline{M}}$ potassium acetate (pH 4.5) and the optical density was read at 360 mμ. From these determinations the first order plots were prepared which extrapolate to a dead time (quenching time) of approximately 3 milliseconds. The pseudo first order constant at 0.237 $\underline{\underline{M}}$ NaOH is 11.7 $sec.^{-1}$ at 25° and the second order constant for the hydrolysis reaction is 49.4 $\underline{\underline{M}}^{-1}$ $sec.^{-1}$. This rate constant is in good agreement with comparative measurements carried out with the optical stopped flow apparatus.

The following conditions cause deterioration in the results obtained:

1. Increase in alkali concentration; this causes difficulties in mixing, streamlined flow and increased quenching time;

2. Wider bore tubing; streamlining;

3. High protein concentration; effects similar to (1).

It is of course evident that the quality of mixing and turbulent flow depend on the work performed on the system. The quality of the engineering, which determines the permissible rate of flow will also determine the applicability of this procedure to more viscous systems. In this respect the demands of the method are not different from the optical stopped-flow device.

As pointed out above, the experiments were performed to test the limitations of the simplest apparatus. The two main improvements which are obviously necessary for the study of enzyme reactions at high protein concentrations are multijet mixing chambers and improved mixing with the quenching solution. We were in fact surprised about the rate of quenching obtained by simple injection from the capillary even without stirring. Stirring did, in fact, often cause slower quenching due to vortex formation. This could be improved by fixing smaller jets at the end of the tube or possibly by ultrasonic treatment, again a case of performing more work on the system.

The equipment as described above has so far been used for the study of two enzyme reactions. First, the rate of dephosphorylation of phosphorylphosphatase which is discussed in detail in section III. Secondly, the rapid quenching technique was also used to correlate the rate of alcohol liberation during enzyme-catalysed ester hydrolysis. The alcohol concentration was determined with alcohol dehydrogenase. This method allows one to follow the rate of alcohol liberation during the first turnover of the enzyme and to correlate this with physical observations in the stopped flow apparatus.

The results obtained from the study of the reaction of chymotrypsin with furylacryloyl-L-tyrosine ethyl ester (FATEE) serve as an example of a number of investigations of this type. This enzyme is specific for the hydrolysis of tyrosine peptides or esters. The N-acylation of the tyrosine with furylacrylic acid provides a useful chromphore, sensitive to any structural re-arrangement at the active site and yet sufficiently far removed from the hydrolysable bond. Spectral changes of this chromophore can be used to follow a number of steps during the reaction of enzyme with substrate. In the same reaction the quenching technique has been used to determine the relative rates of ethanol and acid liberation during the first turnover of the chymotryptic ester hydrolysis. The first order plot in Fig. 1 gives a record of the rate of ethanol liberation, which is approximately 5 times faster than the steady-state rate of acid liberation under the conditions indicated.

The combination of optical observations and chemical sampling has given evidence for the following steps in chymotrypsin-catalysed reactions: (1) Rapid diffusion-controlled adsorption of the substrate to form the Michaelis complex; (2) A structural re-

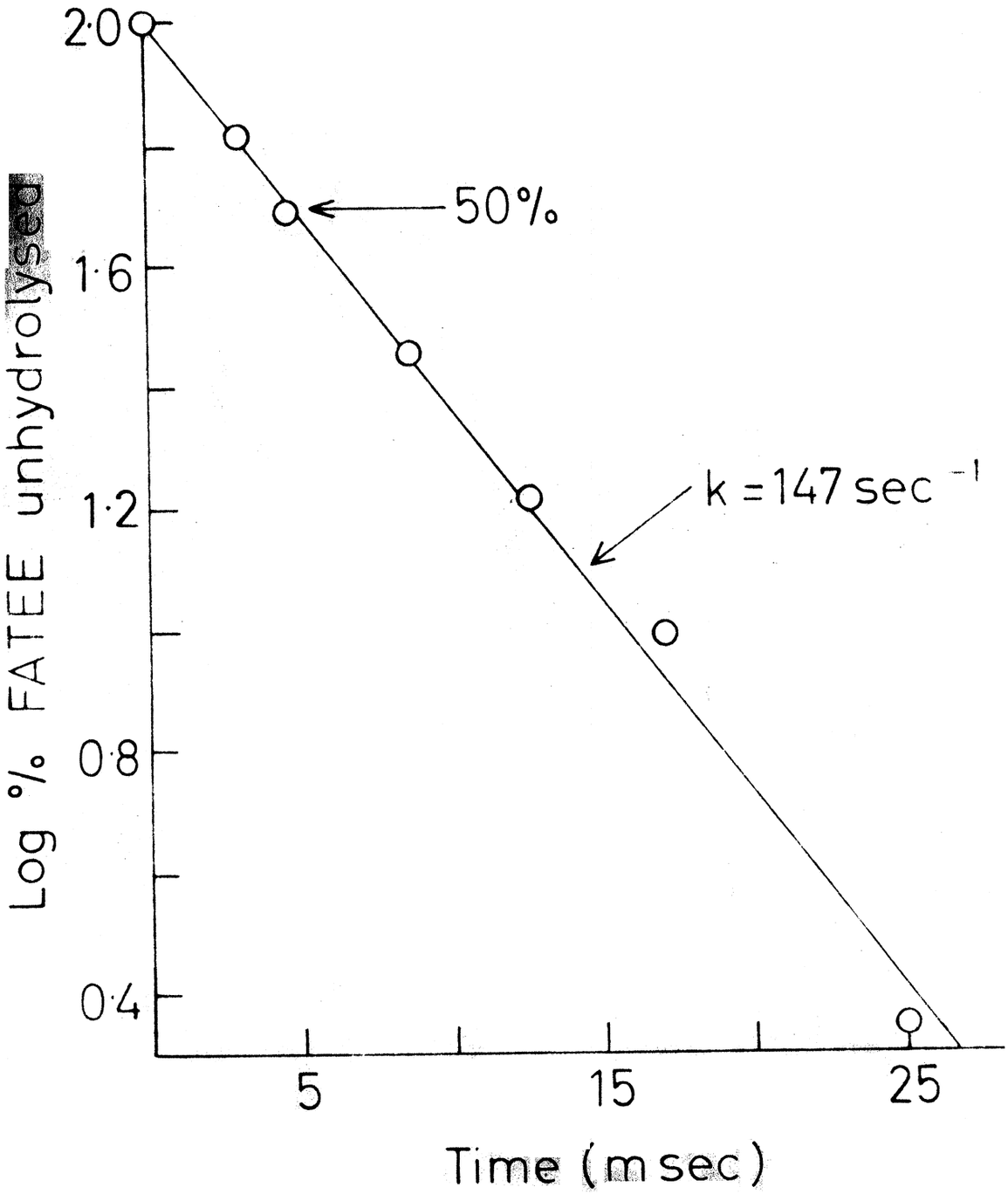


Fig. 1. First order plot showing the rate of liberation of ethanol from FATEE by chymotrypsin hydrolysis. Conditions: 0.270 mM chymotrypsin plus 0.224 mM FATEE plus 0.08 M phosphate, pH 6.6.

arrangement at the active site, which probably transfers the substrate into a hydrophobic crevasse (spectral evidence); (3) Chemical attack by active site histidine and serine which labilises the ethanol residue; (4) Release of product.

The proteolytic enzymes have considerable similarities with the phosphatases with respect to composition of the active site and reaction mechanisms. This is discussed further in the next section.

(III) PHOSPHATASES

(1) The state of phosphatases in milk.

In some preliminary investigations attempts were made to distinguish between different phosphatases which might occur in milk and thus complicate the study of any one of them. In particular we were interested in the question whether there is any specific glucose-6-phosphatase present. This enzyme is present in large amounts in mammalian liver and is of great metabolic importance. We were able to show that there is no glucose-6-phosphatase in bovine mammary gland or milk. This incidentally explains why there is apparently a separate pool of glucose and of glucose-6-phosphate in the mammary gland. In the liver the two compounds are readily interconverted, whereas in the mammary gland glucose can be converted to glucose-6-phosphate (by ATP and hexokinase) but glucose-6-phosphate is not readily converted into glucose.

Cyanide ion inhibits alkaline phosphatase completely without any appreciable effect on acid phosphatase or glucose-6-phosphatase. By this distinction the latter enzyme was found in liver microsomes but not in mammary gland or milk. Attempts to differentiate between acid and alkaline phosphatase by means of selective inhibition with beryllium ions present some interesting complications. The relation between Mg^{2+} and Be^{2+} in the activation and inhibition of enzymes involved in phosphate metabolism is being further investigated at present. The conclusion from preliminary experiments is that at approx. 1.5×10^{-5}M-Be^{2+} the activity of alkaline phosphatase is halved without any significant effect on acid phosphatase or glucose-6-phosphatase. Though there is no glucose-6-phosphatase in milk there is a considerable amount of acid phosphatase. This latter enzyme appears to be present in more constant amounts than alkaline phosphatase which is subject to seasonal and physiological variations. At neutral pH the effective activity of acid phosphatase can often be equal to or greater than that of alkaline phosphatase. It is therefore of importance to study acid as well as alkaline phosphatase from the point of view of its effect on milk constituents. One must also bear in mind that the pH optimum of an enzyme is often dependent on the substrate.

The manner in which alkaline phosphatase is bound to particles in milk is still not understood. That the enzyme is associated with some particulate matter is quite clear (Morton, Biochem. J., 55, 786: 1953; Robert & Polonovski, Discussions Faraday Soc., 20, 54: 1955). Presumably the highly purified enzyme (Morton, Biochem. J., 55, 795: 1953) is free, although its molecular weight has not been reported.

We have investigated the behaviour of native and purified milk

alkaline phosphatase on columns of Sephadex G200. When raw buttermilk was chromatographed, the phosphatase was excluded suggesting that the enzyme (i) has a very large molecular weight (not less than 10^6) (ii) is bound to a complex or (iii) is strongly acidic and thus is repelled by the slightly acidic Sephadex gel.

According to Morton (1953) milk alkaline phosphatase is freed from its complex when treated at room temperature with butan-1-ol. Consequently, raw buttermilk was treated with this reagent, the enzyme precipitated with acetone and subjected to gel filtration on a Sephadex G200 column. Two peaks showing alkaline phosphatase were eluted; peak A (4.5% of recovered activity) being rejected and peak B (95 5%) behaving as a protein of molecular weight in the range 100,000-200,000 A portion of peak B was rechromatographed together with thyroglobulin (molecular weight 670,000) γ-globulin (which behaves as a typical globular protein of mol. wt. ~ 200,000) and bovine serum albumin (67,000) to act as molecular weight standards. The results of this experiment showed that milk alkaline phosphatase was eluted just after γ-globulin, suggesting that phosphatase behaves as a protein of molecular weight 190,000 on a column of G200 Sephadex. Further information and references on gel filtration are given in section (IV).

When peak A was treated with butan-1-ol and the product subjected to chromatography on Sephadex G200, two enzymically active peaks were again obtained - one (75%) corresponding in position to peak A and the other (25%) to the free enzyme (peak B). These results indicate that in raw buttermilk alkaline phosphatase is associated with at least two complexes, one being more stable to butan-1-ol than the other. Methods for separating the complexes were now considered. It seemed likely, in the presence of Mg^{2+} and at high pH that cellulose phosphate might behave as a substrate for the phosphatase which might then be reversibly bound to the ion-exchanger. When a portion of the partially purified enzyme was subjected to chromatography on a column of cellulose phosphate only about 10% of the enzymic activity was eluted as one discrete peak (probably corresponding to a complex) with the developing buffer, but further activity could not be recovered either by increasing the salt concentration or by lowering the pH of the eluting buffer. When p-nitrophenyl phosphate was incorporated into the buffer, the eluate was yellow showing the enzyme to be still absorbed on the column. The phosphatase did not dephosphorylate cellulose phosphate under the conditions used since no inorganic phosphate could be detected in the eluate.

When raw buttermilk was applied to a cellulose phosphate column, two peaks showing enzymic activity were eluted. These were of equal magnitude and represented a total recovery of about 60% of the enzyme applied to the column. Little enzymic activity remained in the column since when p-nitrophenyl phosphate was incorporated in the buffer the eluate remained colourless.

Our results suggest that in buttermilk alkaline phosphatase is attached to at least two complexes which can be separated on a column of cellulose phosphate. The free enzyme, however, becomes firmly bound to the column material. From the results obtained with Sephadex G200 columns it can be inferred that the complexes differ in stability towards butan-1-ol. We are now looking for further differences in the properties of these complexes.

Preliminary molecular weight estimations on milk acid phosphatase were frustated because the enzyme is adsorbed on Sephadex columns. This is not wholly unexpected in view of its known basicity (Bingham & Zittle, Arch. Biochem. Biophys.- 101, 471:1963). We are attacking this problem by subjecting the enzyme to gel filtration on columns of Sephadex G100 and G200 and also of acrylamide (8% 5% crosslinkage) all previously equilibrated with buffers of high ionic strength. These experiments may also provide information as to whether this enzyme is free or particulate in milk.

(2) Studies on Purified Phosphatase from Escherichia coli.

From the studies of Engstrom, Lipmann and Schwartz (for references see Schwartz, Proc. Nat. Acad. Sci., 47, 1996:1963) it is known that incubation of various phosphatases with orthophosphate at slightly acid pH causes phosphorylation of a specially reactive hydroxyl group of the enzyme. It was felt that the study of the reaction of milk phosphatases with radioactive phosphate (^{32}P) would yield some useful information about the active site concentration turnover rate and mechanisms of these enzymes even though they are only available in small amounts and in partially purified form.

The phosphatase induced in E. coli by starving the organism of orthosphosphate is commercially available and has similarities in many respects to the reactions of the mammalian enzymes. It was decided therefore to gain some experience with the phosphorylation reaction using the bacterial enzyme. The specific labelling of the special enzymic serine residues with (^{32}P) can be distinguished from the phosphorylation of other proteins by its rate and the pH dependence of the extent of the reaction. Only one out of approximately 20 serine residues of the enzyme molecules has this specific reactivity for phosphorylation. The maximum rate and extent of phosphorylation occurs around pH5 when the enzyme is catalytically inactive, while at pH 10 when the enzyme is fully active, little or no labelling occurs and phosphoryl-enzyme is rapidly dephosphorylated. Analyses of hydrolysis products of the acid denatured labelled portin yielded O ^{32}P - phosphorylserine. Schwartz (1963) presented circumstantial evidence that phosphorylation with orthophosphate at pH 5 occurred at the active site.

The present investigation was designed to determine the rate of dephosphorylation of phosphorylphosphatase under conditions when the enzyme was active. The rapid-flow quenching technique (Section II 3) used allows the study of reactions with half-times of the order of 5 msec.

E. coli phosphatase (Worthington Biochemical Corp. Freehold N.J. U.S.A.) was obtained in the form of a suspension of 2.5 mg. of protein ml of 0.65 saturated ammonium sulphate solution. For phosphorylation 0.2ml. of this enzyme suspension was added to 1.3 ml. of medium: 0.05 M-sodium acetate-acetic acid buffer pH 5.4 containing magnesium acetate (33mM) and ^{32}P -orthophosphate (1.0 mM: 0.025 mC). The rate of combination of ^{32}P orthophosphate with the enzyme is first-order (0.046 $sec.^{-1}$ at 0°). The ^{32}P -phosphoryl-enzyme was used within 3 hr. of preparation. Prolonged incubation under these conditions leads to a gradual increase of ^{32}P orthophosphate bound. For the study of the rate of dephosphorylation the above solution of

phosphoryl-enzyme at pH 5.5 was mixed in the rapid-flow system with an equal volume of either the same acetate buffer or 0.1 M-sodium carbonate-bicarbonate containing bovine serum albumin (6mg./ml.) to give final pH 5.5, 7 3 or 8.4. After specified flow times (see Fig. 2) the reactions were quenched by rapid mixing of the reaction mixture with 7 N-perchloric acid. The kinetic experiments were carried out at 20°. The phosphorylated protein was isolated by centrifuging and separated from contaminating ^{32}P orthosphosphate essentially by the method of Schwartz & Lipmann (1961). The rate of dephosphorylation was calculated from the decrease in the percentage of ^{32}P-labelled enzyme during the time between mixing the phosphoryl-enzyme solution with the appropriate buffer and quenching in perchloric acid. The blank values without enzyme were about 10% of the maximum bound during several hours at room temperature.

In Fig 2 the results are shown as first-order plots of the data from experiments carried out at pH 8.4 and 7 3. The half-time of the dephosphorylation at pH 8.4 is 6 msec. which corresponds to a first-order constant 115 sec^{-1} and at pH 7.3 the half-time is 43 msec corresponding to a rate constant of 16 sec^{-1}. If the model (Schwartz 1963):

$$E + ROP \underset{}{\overset{k_1}{\rightleftharpoons}} EROP \overset{k_2}{\rightleftharpoons} EP + ROH$$

$$EP \xrightarrow{k_3} E + P$$

is used to illustrate the proposed mechanism of hydrolysis of the ester (ROP) to ROH and orthophosphate (P) catalysed by the enzyme (E). forming the phosphorylated intermediate EP one can draw the following conclusions: at pH 8.4 and 20° k_3 is 115 $sec.^{-1}$ and the overall turnover of p-nitrophenyl phosphate by the enzyme (k_o) is approx. 30 $sec.^{-1}$. Using the kinetic scheme suggested for such a mechanism by Gutfreund & Sturtevant (1956) one can calculate k_2 as 41 sec^{-1} from $k_2 = k_o k_3/(k_3-k_o)$. The precise values for the rate constants will need revision when more accurate information is obtained about the turnover per mole of pure enzyme under the conditions of pH temperature and buffer composition of the present experiment. It should be noted that the first-order constant k_3 may be even larger in the absence of orthophosphate, particularly so at pH 7 3. The qualitative picture shows therefore that the rate constant for the dephosphorylation of phosphoryl-phosphatase is consistent with the proposed mechanism for this enzyme which involves its transient phosphorylation.

(3) The Catalytic Centre activity of Milk Phosphatase.

Several attempts are reported in the literature for devising procedures to obtain pure alkaline phosphatase from bovine milk in reasonable quantities (for refs. see Lyster & Aschaffenburg J.Dairy Res. 29, 21; 1962). None of the methods described yields enzyme in the form of a pure protein or in quantities suitable for conventional physicochemical studies. We have applied the technique of specific labelling to investigate the active site concentration in small samples of partially purified milk phosphatase.

Partial purification of milk alkaline phosphatase. The enzyme was prepared by a modification of the method of Lyster and Aschaffenburg (1962). Raw buttermilk (24 l.) was treated in

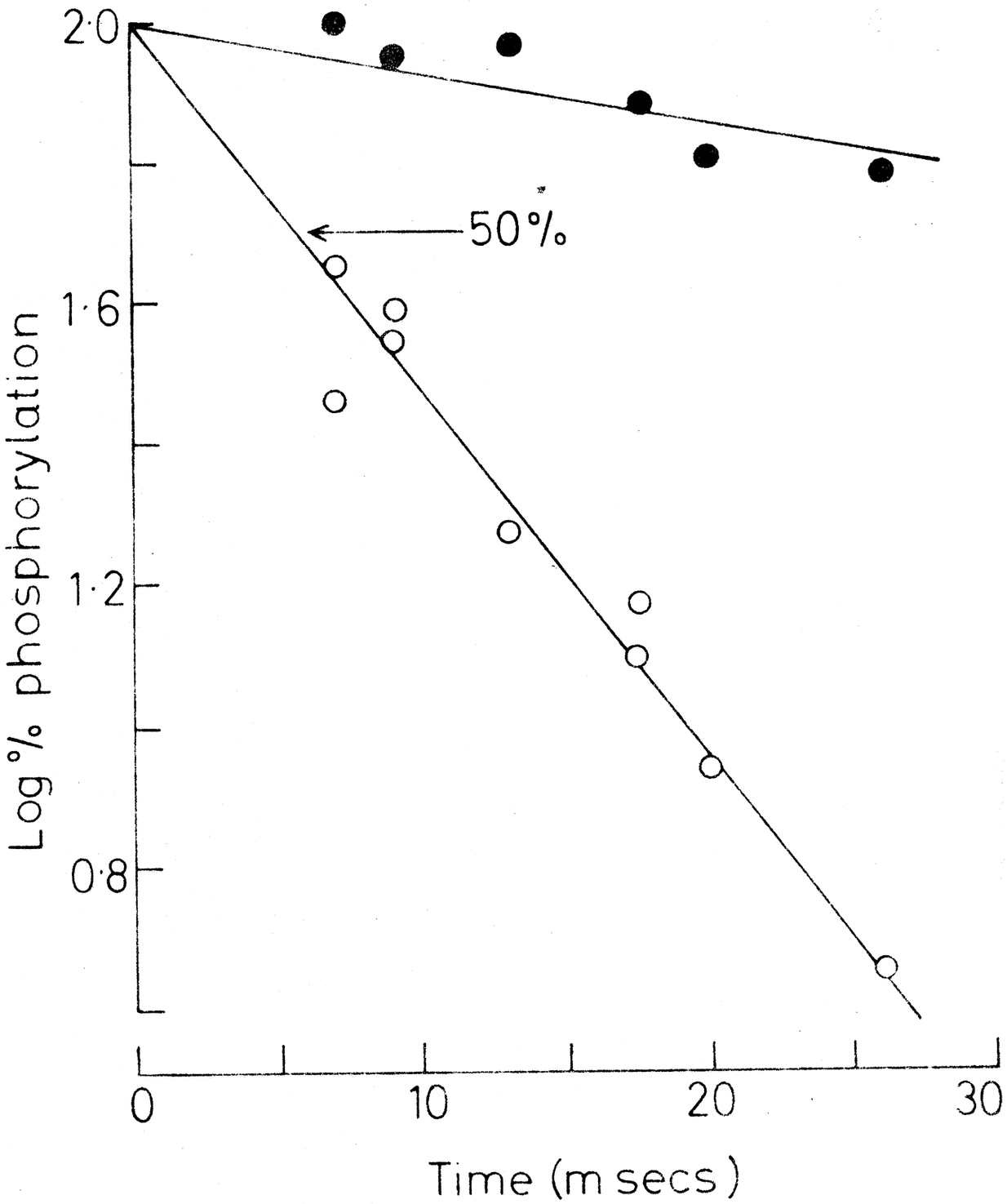


Fig. 2. First order plot showing the rate of dephosphorylation of ^{32}P-phosphorylphosphatase (E. coli enzyme). •, pH 7.5. ○, pH 8.4.

batches of 3ℓ. at pH 6.6 and at room temperature with 1.2 ℓ. of n-butanol and then adjusted to pH 5.0 with 0.25M-acetic acid. The serum was filtered through Hyflo-super cel and after adjustment to pH 8.5 by the addition of 0.2N-NaOH, left at 4.5° overnight.

The supernatant was collected by decantation, adjusted to pH 6 3 and ice-cold acetone added to 50% saturation (v/v). The mixture was left overnight at room temperature and the resulting precipitate collected and extracted with 50 mM-magnesium acetate (3 x 200 ml.). After centrifugation the combined extracts were fractionated with ice-cold acetone and the precipitate from the 33⅓-50% saturated solution retained. The precipitate was dissolved in 50 mM magnesium acetate (15ml.) and excess acetone removed by freeze-drying Little activity was lost by this treatment. The residue was extracted with 15 ml. of water and centrifuged at 105,000g. for 60 min. to yield a red solution containing 8 8mg./ml. of protein as determined by the micro-Kjeldahl method. The specific activity of the preparation was 31 µ moles p-nitrophenyl phosphate hydrolyzed /min./mg. protein at pH 10.0.

Partial purification of milk acid phosphatase. The enzyme was prepared by a modification of the method of Bingham & Zittle (1963). Raw skim milk (50l.) was stirred with 350g. Amberlite IRC-50 (ammonium form) ion-exchange resin for 1 hr. at room temperature. The resin was collected on a sintered glass funnel and washed with water until the filtrate was clear. The enzyme was eluted with 1M-ammonium acetate (pH6.35, 5 x 200 ml.) The extract was clarified by centrifugation, the supernatant fractionated with ice-cold acetone and the precipitate from 46-73% saturation retained. The precipitate was dissolved in water (32 ml.). to yield a green solution containing 6.3 mg. of protein/ml. The specific activity of the preparation was 0 36 µmoles p-nitrophenol phosphate hydrolyzed/min./mg. protein at pH 5.2.

Alkaline phosphatase assay. Alkaline phosphatase activity was estimated using p-nitrophenyl phosphate as substrate. At pH 7 5 and above, 0.05M-sodium carbonate-bicarbonate was used as buffer; at pH7 0 and below, sodium acetate-acetic acid was employed In all cases the reaction mixture contained 5mM-p-nitrophenyl phosphate and 1mM-magnesium acetate The hydrolysis was observed continuously at pH6.5 and above. At pH values lower than 8.5 the observed optical densities were corrected for; the pK of p-nitrophenyl was assumed to be 7.0 At pH6 0 and below the enzyme was assayed as for acid phosphatase by the method of Bingham & Zittle (1963)

In certain experiments β-glycerophosphate was used as substrate. The final concentrations of the reactantswere 10mM- β-glycerophosphate 1mM-magnesium acetate and 0.05M-sodium carbonate - bicarbonate buffer pH 10.0. At appropriate time intervals the reaction was stopped by the addition of 0.5ml 24 N-H_2SO_4 and the concentration of orthophosphate determined by the method of Briggs (1932). One alkaline phosphatase unit is defined as that amount of enzyme which will hydrolysze 1 µmole p-nitrophenylphosphate/min. at pH10.

Acid phosphatase assay. Acid phosphatase activity was assayed by the method of Bingham & Zittle (1963) One acid phosphatase unit is defined as that amount of enzyme which will hydrolyse 1 µmole p-nitrophenylphosphate/min. at pH5.2.

Partial purification of milk alkaline phosphatase phosphoryl-enzyme. In a typical experiment, 27 units of alkaline phosphatase (0.88mg. protein in 0.1ml. 50mM-magnesium acetate solution) was added to a reaction mixture (0.9ml.) containing 2 x 10^7 counts per minute carrier free (^{32}P)-orthophosphate, 0.2mM-sodium orthophosphate and buffer according to the pH of the experiment (see alkaline phosphatase assay section). The solution was kept at 0° for 1 min., after which time the reaction was quenched by the addition of 20% perchloric acid (0.2ml.) and the resulting precipitate collected by centrifugation. The pellet was washed with 5% perchloric acid (5 x 6ml.) and dissolved in 90% formic acid (0.5ml.). Water (4.5ml.) was added and the mixture centrifuged. The phosphorylenzyme was precipitated from the supernatant by the addition of 20% perchloric acid (1.0ml.) and collected by centrifugation followed by washing with 5% perchloric acid (3 x 6ml.) The final pellet was dissolved in 90% formic acid (1.0ml.) and a portion (0.5ml.) withdrawn and placed on a planchette. The acid was removed by gentle heating on a hot plate (80-90°) and the residual activity counted (Isotope Developments Ltd. Scintillation Counter). The efficiency of the counter was 49%.

pH Optima of phosphorylation and of p-nitrophenyl-phosphatase activity. The pH optima of phosphorylation and of p-nitrophenyl-phosphatase activity are shown in Fig. 3 and are 5.1-5.2 and 9.9-10.1 respectively.

Incorporation of (^{32}P)-orthophosphate into milk alkaline phosphatase at pH 5.0 or pH 7.0. When alkaline phosphatase was incubated with varying concentrations of (^{32}P)-orthophosphate at pH 5.0 and the phosphoryl enzyme isolated as described above, the slope of the line gave the concentration of alkaline phosphatase active sites used as 1.695 x 10^{-7}M. Since 27.6 enzyme units were employed, the catalytic centre activity of the enzyme is 2722 $sec.^{-1}$ with p-nitrophenyl phosphate or 2240 $sec.^{-1}$ with β-glycerophosphate as substrate. If it is assumed that the enzyme has one active site per molecule of molecular weight 190,000 (see above) its specific activity is 860 (p-nitrophenyl phosphate as substrate). The purity of the preparation used was thus 3.5%.

At pH 7.0, 27.6 enzyme units (or 1.695 x 10^{-7}M) were incubated with increasing concentrations of (^{32}P)-orthophosphate. If the phosphorylation is interpreted in terms of mechanism A, values of Ki and Kp of 1.94 10^{-4}M and 0.50 respectively, are obtained assuming Eo = 1.70 10^{-7}M). If, however, mechanism B is assumed to be correct, a value for (Eo) of 6.85 x 10^{-8}M is obtained - or about 40% of that actually used.

Effect of denaturation on the incorporation of (^{32}P) orthophosphate into milk alkaline phosphatase. Samples (0.7ml.), each of which contained 27 units of milk alkaline phosphatase (8.8 mg. protein) and 0.066 M sodium acetate-acetic acid buffer (pH4.6), were kept at 40°. At appropriate time intervals (1 min. to 1 hr.) they were removed and placed on ice. Portions (0.02 ml.) were assayed for alkaline phosphatase activity at pH 10.0. The increase in extinction was observed at 450 mμ (ϵ = 3100 $M^{-1}cm^{-1}$) rather than 400 mμ (ϵ = 18,200 $M^{-1}cm^{-1}$). The enzyme left in the remaining 0.68 ml. was phosphorylated by the addition of orthophosphate and magnesium acetate to provide a final volume of 1.0 ml. The final concentrations were: 1mM-sodium orthophosphate; 8.5 x 10^6 count per min. (^{32}P)orthophosphate; 0.035 M-magnesium acetate; 0.05 M-sodium acetate-acetic acid buffer pH 5.0. The mixture was kept at 0° for 1 min. and then quenched by the

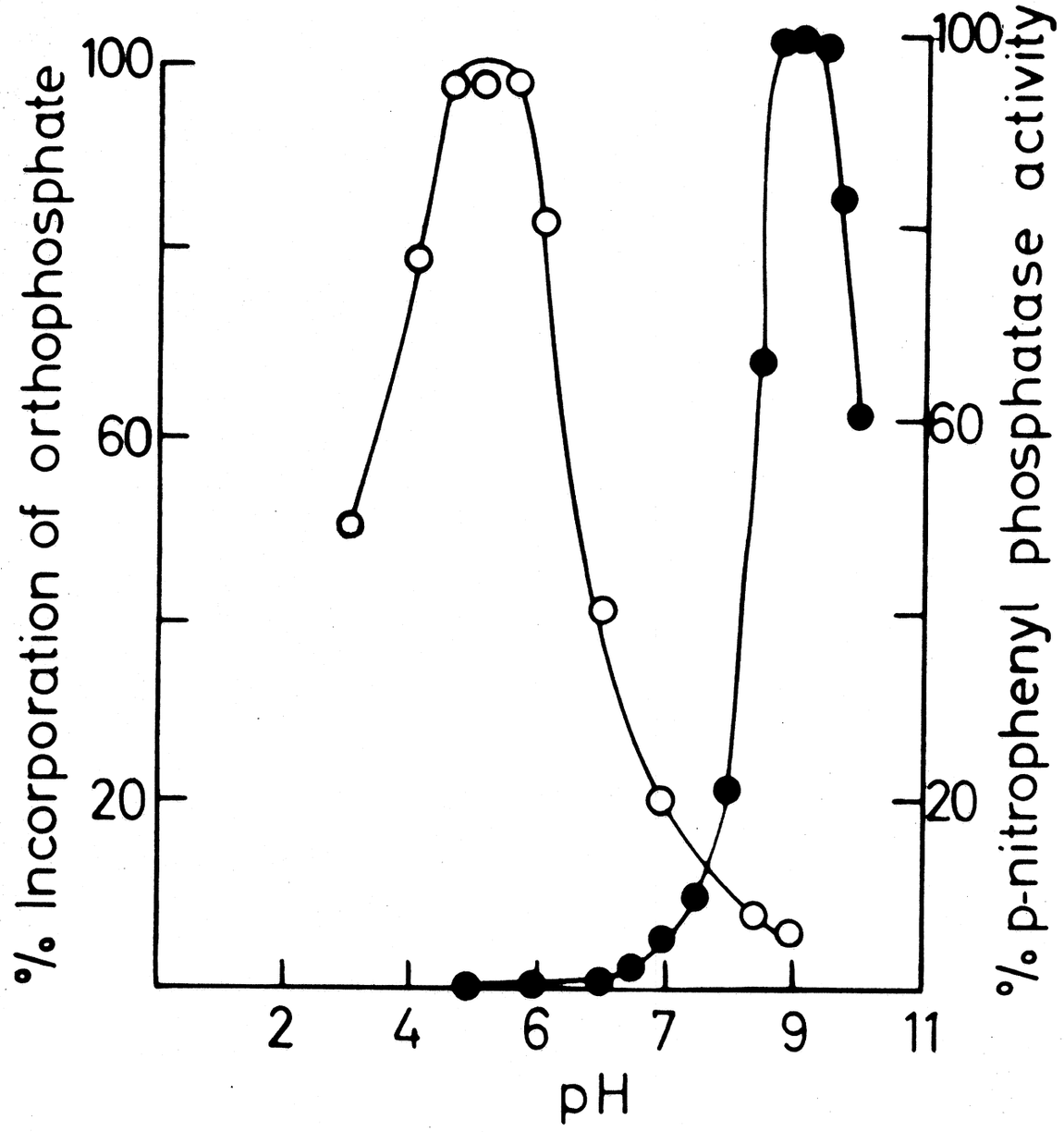


Fig. 3. pH Optima of phosphorylation (o) and of p-nitrophenylphosphatase activity (•) of milk alkaline phosphatase.

addition of 20% perchloric acid (0.2 ml.). The precipitated alkaline phosphatase was separated from excess (^{32}P)-orthophosphate as described above. The enzyme loses its ability to incorporate ^{32}P-orthophosphate when it is progressively denatured and this loss closely parallels its concomitant loss of p.nitrophenyl phosphatase activity.

Dephosphorylation experiments. The phosphorylenzyme was prepared by incubating at room temperature a mixture of 1.7 x 10^{-6}M-(or 276 units) alkaline phosphatase, 8 x 10^{7} counts per minute (^{32}P)-orthophosphate, 2 x 10^{-4}M-orthophosphate, 0.06 M-magnesium acetate and 0.4 M-sodium acetate-acetic acid buffer, pH 5.0. The rate of dephosphorylation of the phosphorylenzyme was studied by mixing the above solution in the rapid flow-quenching apparatus of Barman & Gutfreund (1964) with the same volume of 0.05 M-sodium acetate-acetic acid buffer, pH 5.0, 0.09 N-NaOH or 0.2 M-sodium carbonate buffer, pH 10.0. The buffers contained 0.4 mg./ml. serum albumin. The final pH values obtained were 5.0, 7.0, and 9.6 respectively. The reaction mixture was quenched in 3 ml. of 20% perchloric acid. The precipitated protein was separated from excess (^{32}P)-orthophosphat and counted as described above. When phosphoryl alkaline phosphatase was dephosphorylated in the rapid reaction-quenching machine at pH 7.0, the equilibrium concentration of phosphorylenzyme was in good agreement with the pH optimum profile (Fig. 3). When the results are interpreted according to mechanism A, $k+3 + k-3 = 164$ sec.$^{-1}$. Since $k+3/k-3 = Kp = 0.5$ M, $k+3 = 53$ sec.$^{-1}$ and $k-3 = 110$ sec.$^{-1}$. On the other hand, if mechanism B is assumed to be correct then $k+4 = 119$ sec.$^{-1}$ and since in this case $k+4/k-4 = Ki = 1.1\ 10^{-4}$ M, $k-4 = 1.08 \times 10^{6}$ M^{-1} sec.$^{-1}$

Further kinetic constants: Km for p-nitrophenyl phosphate and Ki for orthophosphate at pH 10.0 & 7.0. At pH 10.0 the constants were readily measured by conventional means: the Km for p-nitrophenyl phosphate was 4.7 x 10^{-7}M and the Ki for orthophosphate 3 x 10^{-4}M. However, the Km for p-nitrophenyl phosphate at pH 7.0 was very small ($\sim 2 \times 10^{-7}$ M) and consequently difficult to measure accurately. The Ki for orthophosphate was approximately 1 x 10^{-4}M. These values are probably of the correct order of magnitude. Both values were obtained from experiments where at different orthophosphate concentrations the p-nitrophenylphosphate concentration was varied thus giving rise to a series of apparent Michaelis constants. A plot of Km (app.) against orthophosphate concentration yielded the required constants. At both pH values, orthophosphate competetively inhibited the hydrolysis of p-nitrophenyl phosphate by the milk enzyme.

Incubation of milk acid phosphatase with (^{32}P)-orthophosphate at pH 5.0. When 0.46 units of acid phosphatase were incubated with 2 x 10^{-3} or 2 x 10^{-4}M (^{32}P)-orthophosphate (as described for alkaline phosphatase) and excess orthophosphate removed, the purified protein obtained therefrom showed very low specific activity.

Table 1. The effect of high ionic strength, tris, glycine or EDTA on a) the incorporation of orthophosphate b) p-nitrophenyl phosphatase activity of.

a) Incorporation of orthophosphate For each incubation 27.6 units of enzyme were incubated with 2.5×10^{7} counts per minute of (^{32}P)-orthophosphate, 1×10^{-3} M-sodium orthophosphate, 3.5×10^{-2} M magnesium acetate, $5\ 10^{-2}$ M-sodium acetate-acetic acid buffer, pH 5.0, and NaCl, tris, glycine or EDTA. The mixtures were kept at 0° for 1 min. and the enzymes separated from excess (^{32}P)-orthophosphate and counted as described in the text.

b) p-Nitrophenyl phosphatase activity. In each experiment 2.76×10^{-2} units (or 1 µg) or enzyme was added to a mixture containing 5×10^{-3} M-p-nitrophenyl phosphate, 1×10^{-3} M -magnesium acetate, 5×10^{-2} M-sodium carbonate-bicarbonate buffer, pH 10.0 and NaCl, tris or glycine.

Addition	Recovery of phosphoryl enzyme	Recovery of activity
none	1.00	1.00
0.5 M-NaCl		1.32
0 8 M-NaCl	0.75	1.43
1.0 M-NaCl	0.72	1.50
0.5 M-tris		2.60
1.0 M-tris	0.25	
0.5 M-glycine		0.11
0.8 M-glycine	0.28	
$5\ 10^{-3}$ M-magnesium acetate(*)	0.85	
$3.5\ 10^{-2}$ M-magnesium acetate(*)	1.00	1.00
$6\ 10^{-2}$ M-magnesium acetate (*)	0.99	
$9\ 10^{-2}$ M-magnesium acetate (*)	0.91	
$5\ 10^{-2}$ M-EDTA (†)	0.59	

* These are total concentrations.

† The incubation mixture contained 5×10^{-3} M-magnesium acetate.

Discussion. Our experiments demonstrate that milk alkaline phosphatase in crude preparations can be specifically phosphorylated. Thus, glucose 6-phosphate or p-nitrophenyl phosphate (1×10^{-4} M) abolished the incorporation and in denaturation experiments the loss of p-nitrophenyl phosphatase activity closely paralleled the concomitant loss of ability to incorporate (^{32}P)-orthophosphate.

The origin of the slight incorporation of (^{32}P)-orthophosphate in the absence of enzyme activity is a moot question. It is known that human prostate and potato acid phosphatases incorporate orthophosphate, albeit to lesser extents than does the alkaline phosphatase. Consequently, our alkaline phosphatase preparation was assayed for phosphatase at pH 5.0, in the presence of 10^{-2} M sodium cyanide (cyanide inhibits alkaline phosphatase strongly whereas acid phosphatase is unaffected). It was found that the p-nitrophenyl phosphatase activity of the preparation at pH 5.0 was entirely due to acid phosphatase and that the amount of this enzyme was about 0.08% (expressed as a percentage of the alkaline phosphatase activity.) Since the ability of acid phosphatase to incorporate (^{32}P)orthophosphate is low, the presence of this small amount of the enzyme in the alkaline phosphatase preparation cannot explain the extra alkaline phosphatase incorporation. The residual incorporation - absent in the presence of substrate - is, therefore, difficult to explain.

Milk alkaline phosphatase has a catalytic centre activity of 2722 $sec.^{-1}$ with p-nitrophenyl phosphate as substrate. This must be a maximum figure for it assumes that the phosphorylation of the enzymes is complete under the conditions used. It is of interest to note that the molecular activity of the bovine liver enzyme is about 2640 sec^{-1}. Furthermore, both enzymes behave on Sephadex G-200 columns as proteins of molecular weight about 190,000 The catalytic efficiency of these enzymes - in common with other non-bacterial alkaline phosphatases - is much greater than that of the bacterial enzyme. For example, the E. coli enzyme has a molecular activity of about 35 sec^{-1}. suggesting that the E. coli enzyme lacks a histidine residue at its active centre. It is tempting to suggest that the enhanced activity of the animal enzymes is due to the presence of this amino-acid at their catalytic centres.

Comparing the data obtained here for the specific activity per catalytic centre with activities obtained for purified enzyme, it is found that the most active preparation of the bovine milk enzyme (Lyster & Aschaffenburg loc. cit.) contained 57.5% enzyme.

Another noteworthy conclusion from our studies is that although the optimum catalytic rate of milk alkaline phosphatase is at pH 10, the Michaelis constant is much more favourable at neutral pH. At very low substrate concentration the enzyme is more efficient at neutrality.

IV MILK LIPASES AND THEIR INTERACTION WITH CASEIN MICELLES.

Experiments with various substrates and enzyme inhibitors have provided evidence for the presence of mixtures of lipases or other esterases in milk (Frankel & Tarassuk, J. Dairy Sci. 40, 1517, 1523: 1957; Forster, Bendixen & Montgomery, J. Dairy Sci. 42, 1903: 1959; Forster, Montgomery & Montour, J. Dairy Sci. 44

1420:1961) but the individual enzymes in such a mixture are difficult to recognize and characterize in the presence of one another because of their low, overlapping substrate specification. Efficient fractionation methods which can be applied to crude preparations will clearly be helpful for the investigation of these enzymes, and in the expectation that milk lipases differ from one another in molecular weight the technique of gel filtration has been applied to their study. Good correlation exists between the gel filtration behaviour and the molecular weights of many enzymes and other globular proteins (see Andrews, Biochem J. 91, 222:1964; 96 595:1965 and references therein) so, besides being a means for separating lipases, gel filtration may enable their molecular weights to be estimated. In addition, the gel filtration technique provides a means of investigating the interaction between milk lipases and casein micelles.

(1) Lipase assay.

After some preliminary investigation of manometric and colorimetric methods the method eventually chosen for lipase assay was the automatic titration of the acid liberated from tributyrin emulsion substrate, or from other substrates when specificity studies were being carried out. Tributyrin was chosen as the substrate for general work because, to judge from the literature, it is likely to be hydrolysed by most lipases at a satisfactory rate. Tributyrin emulsion was prepared by homogenizing the substrate (10 ml.) and gum acacia (10 g) in ice-water (80 ml.) adjusting the pH to 8.5, and diluting to 100 ml. The emulsion was used only on the day of preparation.

Continuous titration was performed with a Radiometer titrator type TTT1c coupled to a Radiometer titrigraph type SBR2C. The reaction occured at 25° in a water-jacketed vessel (inner dimensions 2.3 cm. diam. x 7.7 cm. deep) containing up to 12 ml. of assay mixture, which included 2 ml. of substrate emulsion. The assay mixture was continuously stirred and bubbled with CO_2-free N_2 (250 ml./min). Assays with tributyrin as substrate were carried out at pH 8.5, but the enzyme-substrate mixtures were first adjusted to pH 8.7 with alkali from the micro-syringe of the titrator, and recording of the titration was not commenced until the pH of the mixtures had dropped to the pre-set value. Rates of acid production recorded in this way were linear with time, and proportional to the amounts of enzyme added. Lipase in solutions of low ionic strength was assayed satisfactorily only if salt (1 ml. of 0.1 M-NaCl) was added to the assay mixture. With 0.001 N-NaOH as titrant, the method was used to measure rates of acid liberation in the range 10-70 mμ equiv./min, allowing lipase assays on as little as 0.02 ml. of milk. To assay the activity of larger milk samples (up to 5 ml.), 0.01 or 0.1 N-NaOH was used.

One unit (U) of lipase activity is taken as the amount of enzyme liberating 1 μequiv. of acid from the substrate/min.

(2) Lipase activity in milk and skim milk.

Estimation of the lipase activity in milk and skim milk was combined with an investigation of the conditions necessary to elicit maximum activity.

Lipase activity in whole milk, measured on 10 ml. samples with tributyrin emulsion as substrate (total assay volume 12 ml.),

was generally in the range 0,2-0.4 U/ml. Up to 15% of this activity was detected when no substrate was added. Assays on 10 ml. samples of skim milks indicated the presence of 30-50% more activity/ml. than in the corresponding whole milks. No lipase activity was detected in aqueous dispersions of the fat layer obtained by centrifuging fresh raw milk at 80,000 g for 1 hr., but coating of the titrator electrodes by fat rendered the result uncertain. The higher tributyrinase activity of skim milk as compared with that of whole milk is probably not an activation effect resulting from the process of cream separation because Mattick & Kay (J.Dairy Res. 9, 58:1938) found the same thing when the cream was allowed to rise of its own accord. A likely explanation is that the enzymes have kinetic properties and substrate affinities which result in the acid liberation from tributyrin alone as in the assay of skim milk, being more rapid than acid liberation from a mixture of tributyrin and milk fat, as in the assay of a relatively large sample of whole milk. Milk lipases hydrolysed emulsified tributyrin at a faster rate than they hydrolysed milk fat emulsion (Table 2).

Ultracentrifugation of fresh uncooled milks or skim milks at 80, 000 g for 1 hr. sedimented most of the casein and 70% of the lipase (Table 3).

Assays with 0.05 ml. or 10 ml. samples of the supernatant (supernatant (A)) gave the same values for lipase activity/ml, and no increase in the lipase activity was detected when samples were assayed in the presence of 0.75 M NaCl. On the other hand, when assays were performed on 0.05 ml. samples of skim milk (total assay volume, 2.05 ml.) 40-60% more lipase activity/ml. of sample was detected than when assays were performed on 10 ml. samples. In addition, stepwise addition of NaCl to skim milk resulted at first in an increase in lipase activity, but inhibition occurred at salt concentrations above 0.75 M Maximum increases in activity ranged from 40% to 100% when 10 ml. samples were assayed, but were only 5-20% for the same milks when 0.05 ml. samples were assayed. In both cases, the NaCl concentration in assay mixtures was similar to that in the activated samples. The interpretation of these results is that lipases associated with casein micelles are not fully active, but both dilution and addition of sodium chloride are effective in stimulating or restoring their activity, probably by dissociating micelle-lipase complexes (see below). Dilution or sodium chloride addition did not enhance the activity of high-speed supernatant preparations which contained little or no micelle-bound lipase.

When assayed under optimum conditions, (0.2 ml. samples in the presence of 0.6 M-NaCl), the lipase activities towards tributyrin emulsion of fresh whole milks from 61 Friesian cows were in the range 0.2 - 1.7 µ/ml. with a mean of 0.88 µ/ml. The values followed a normal distribution, the standard deviation was 0.31 µ/ml, the standard error of means was 0.04 µ/ml. and the coefficient of variation 35%. The results are very similar to those reported by Frankel & Tarassuk (J. Dairy Sci. 39, 1517:1956) and by Dunkley & Smith (J. Dairy Sci. 34, 935, 940:1951) for hydrolysis of tributyrin by milk lipase, but it is surprising that the latter authors reported such high activity because as substrate they used tributyrin solution, which is hydrolysed much more slowly than tributyrin emulsion (Table 3). Lower results were obtained by Forster, Montgomery & Montoure (J. Dairy Sci. 44, 1420:1961) who used a manometric assay method at pH 8.0, and much lower results by Mattick and Kay (J. Dairy Res. 9, 58:1938)

Table 2. Substrate specificity of skim milk lipase

Substrate	Hydrolytic activity of skim milk (U/ml.) Milk 1	Milk 2	Milk 3	Milk 4
Tributyrin emulsion	0.84 (100%)	1.34 (100%)	0.92 (100%)	1.32 (100%)
Triolein emulsion	0.42 (50%)	0.54 (40%)	0.41 (45%)	0.62 (47%)
Milk fat emulsion	0.64 (76%)*	0.74 (55%)+	0.50 (54%)+	0.99 (75%)*
Tributyrin solution	0.11 (13%)	-	-	0.26 (20%)
Triacetin solution	0.10 (12%)	0.11 (8%)	0.10 (11%)	0.13 (10%)

* Milk fat sample (1) + Milk fat sample (2). See the text.

Table 3. Analytical results for skim milk and high-speed supernatants prepared therefrom

Preparation of the high-speed supernatants is described in the text. Tributyrinase activity was estimated on 0.05 ml. samples in each case. Total N and casein N were determined by the method of Rowland (J. Dairy Res., 9, 42: 1938) and sialic acid by the thiobarbituric acid method of Warren (J. Biol. Chem., 234, 1971; 1959).

	Tributyrinase activity (U/ml.)	Total N (mg./ml.)	Casein N (mg./ml.)	Sialic acid (µg./ml.)
Skim milk	-	4.88 (100%)	3.85 (100%)	96.0 (100%)
Skim milk + 0.75 M-NaCl	0.82 (100%)	4.90	3.86	-
Supernatant (A)	0.24 (29%)	1.29 (26%)	0.16 (4.2%)	-
Supernatant (B)	-	1.23 (25%)	0.14 (3.5%)	-
NaCl-Supernatant (A)	0.56 (68%)	1.59 (32%)	0.42 (11%)	-
NaCl-Supernatant (B)	0.53 (65%)	1.27 (26%)	0.14 (3.5%)	1.52 (1.5%)

and Heyndrick & Peeters (Enzymologia, 20, 161:1958) who used tributyrin solution as substrate. There are as yet insufficient results obtained under optimum conditions to show whether or not the lipase activity in milk differs significantly from one breed of cow to another.

The hydrolytic activities of four different skim milks towards five substrates are given in Table 2. Triolein emulsion and milk fat emulsion for these experiments were prepared as described above for tributyrin emulsion, except that triolein (1 ml.) or milk fat (0.5 g.) were used instead of tributyrin. For triacetin solution the triglyceride (1 ml.) was dissolved in water (100 ml.) and the pH adjusted to 8.5. Tributyrin solution was prepared by dispersing tributyrin (1 ml.) in water (90 ml.), centrifuging the resultant suspension at 10,000 g. for 30 min., and diluting the clear solution with an equal volume of water. The pH was adjusted to 8.5. The relative differences in activity towards milk fat are not necessarily due to differences in the lipases in the various milks because gas-liquid chromatographic analyses showed that the fatty acids of milk fat sample (1) contained 20% of palmitic acid whereas those of sample (2) contained 30% of palmitic acid and correspondingly lesser amounts of various other acids. The variations in the relative activities of the individual milks towards the different substrates indicate the presence in them of more than one lipase but the variation is less marked than that reported by Frankel and Tarassuk (J. Dairy Sci., 39, 1517:1956) from a more extensive investigation of this type.

pH Optimum of skim milk lipase. Skim milk lipase showed maximum activity towards tributyrin emulsion at pH 8.7 when assayed at pH's ranging from 5.5 to 11.5 This is in agreement with previous observations (Frankel & Tarassuk, J. Dairy Sci. 39, 1506: 1956), and the other pH optima reported for milk lipases (cf. Chandan & Shahani, J. Dairy Sci., 47 471:1964) were not observed.

Lipase activities of high-speed supernatants from milk. As indicated above, supernatant (A), which contained about 30% of the milk lipase, was prepared by centrifuging skim milk in a spinco model L ultracentrifuge (rotor 30) at 80,000 g for 1 hr. at 2°, and gently decanting the solution. Some slowly-sedimented casein was carried over with the solution. Supernatant (B) was also prepared from skim milk but centrifugation was extended to 2 hr. at 80,000 g and the clear supernatant collected through a hole pierced in the lusteroid centrifuge tube about 1 cm. above the casein pallet. NaCl-supernatants (A) and (B), which both contained about 70% of the milk lipase, were prepared by similar procedures from skim milk to which NaCl (0.75 M final concn.) had first been added. The extinction at 340 mμ (1 cm. cuvette) of NaCl-supernatant (B) preparations was taken as a measure of their casein micelle content, and any with E 340 mμ > 0.2 were discarded. Analyses of these supernatant preparations are given in Table 2.

(3) Milk Lipases and Lipase-casein interaction studied by gel filtration.

The preparation of the milk lipase solutions containing only small amounts of casein (NaCl-supernatants (A) and (B)) opened the way to further investigations, mainly by gel filtration experiments.

Gel filtration techniques. Columns (50 cm. x 2.4 cm. diam.) of Sephadex G-200 were packed in the cold as described by Andrews (Biochem J. 96, 595:1965) and equilibrated with salt

solutions made up in redistilled water as indicated for the various experiments. Effluent was collected in 5 ml. fractions using a collector fitted with a syphon. All columns were run at 0-5°, and flow-rates were 10-15 ml/hr. The columns were calibrated for estimating the molecular weights of proteins as described by Andrews (Biochem J. 91, 222: 1964; 96 595:1965) using glucagon, cytochrome c, chymotrypsinogen, adenosine deaminase, bovine serum albumin, Escherichia coli phosphatase, lactate dehydrogenase, human γ-globulins, apoferritin and E. coli β-galactosidase as molecular weight standards (cf. Andrews loc. cit.).

Effect of sample size on the gel-filtration behaviour of the lipases in NaCl-supernatant (A). Samples of NaCl-supernatant (A), prepared from one milk and ranging in size from 3 ml. to 18 ml. and containing E. coli β-galactosidase, lactate dehydrogenase and E.coli phosphatase as standards were submitted to gel filtration on a column equilibrated with 0.75 M-NaCl plus 0.025 M-$MgCl_2$. The results are shown in Fig. 4.

In order of increasing elution volume, the five peaks in the protein elution diagrams (e.g. Fig. 4c or 4f) probably represent casein micelles, milk globulins + casein aggregates (see under the next heading), β-lactoglobulins, α-lactalbumin and low molecular weight material respectively. Lipase was eluted in similar positions whether 3 ml. or 4 ml. samples were used (Figs. 4e 4f), but it was eluted progressively earlier as the sample size was further increased, and appeared eventually as an asymmetric band of ill-defined peaks. Increasing the sample volume from 10 ml. to 18 ml. effected only a slight change in the elution volume of the main lipase peak (Figs. 4a 4b) and in both cases it was eluted later than the casein micelle peak, in a position corresponding to that of a 'typical' globular protein (cf. Andrews, Biochem J. 96, 595:1965) of molecular weight about 350,000. Recoveries of lipase ranged from 70% for the 18 ml sample to 25% for the 3 ml. sample. Experiments with even smaller samples were unsuccessful because insufficient lipase was recovered for satisfactory assays. The elution volumes of the standards were unaffected by sample size.

Effect of ionic strength on the gel-filtration behaviour of the lipases in NaCl-supernatant (A). NaCl-Supernatant (A) preparations (4 ml. portions) from two different milks were submitted to gel filtration on columns equilibrated with various concentrations of NaCl containing 0.025 M-$MgCl_2$. The results are shown in Fig. 5.

The interpretation of the protein elution diagrams in Figs. 5a and 5e is the same as for Fig. 4., but in addition the presence of β-casein aggregates (apparent mol. wt ~ 200,000) in the second peak (V_e ~ 100 ml.? Fig 5a) was indicated by the development of opalescence (measured by extinction at 340 mμ) in this region when the temperature of fractions from the runs at lower ionic strengths rose to 25°. β-Casein is much more soluble in the cold than at 25° (Waugh, J. Phys. Chem. 65, 1793 :1961). The non-appearance of opalescence in this region in fractions from runs in 0.75 M-NaCl + 0.025 M-$MgCl_2$ is presumably due to a greater solubility of β-casein under such conditions. The opalescence associated with the casein micelle peak showed no change with temperature.

The major part of the lipase activity of each preparation was eluted with the casein micelles from columns equilibrated with 0.05 M-NaCl + 0.025 M-$MgCl_2$ (Fig. 5b) whereas typical milk lipase elution diagrams with very little enzyme in the casein

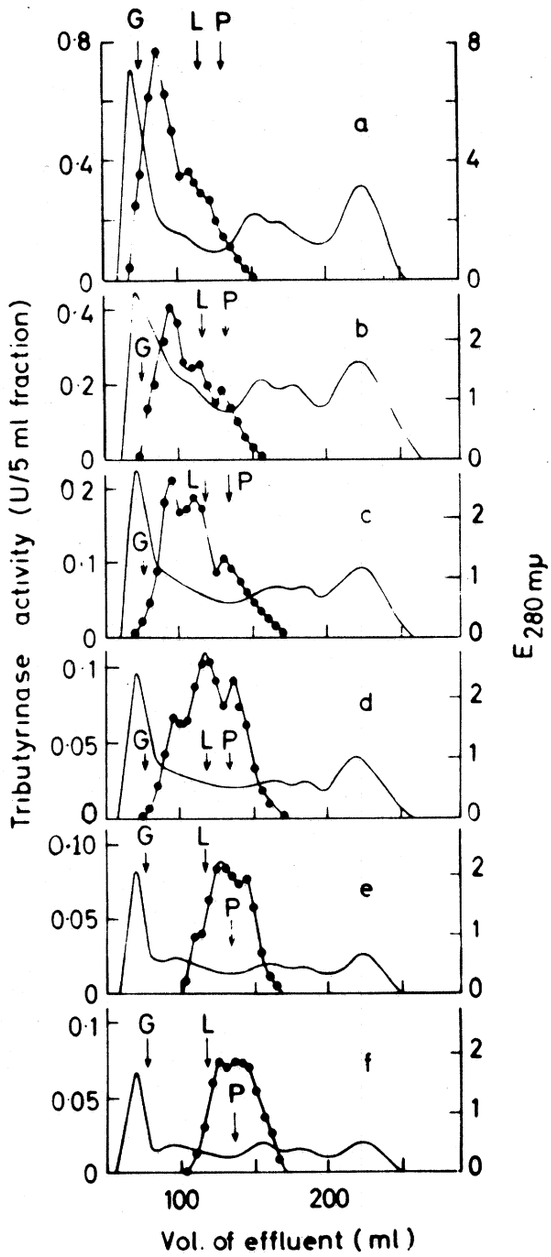


Fig. 4. Effect of sample size on the gel filtration behaviour on Sephadex G-200 columns of the lipase in NaCl-supernatant (A), prepared from skim milk as described in the text. Volumes of NaCl-supernatant (A) used were (a) 18 ml.; (b) 10 ml.; (c) 6 ml.; (d) 5 ml.; (e) 4 ml.; (f) 3 ml. ——, Extinction at 280 mμ; ●——●, lipase activity. G, L, P and the arrows indicate the elution volumes of E. coli β-galactosidase, lactate dehydrogenase and E. coli phosphatase respectively, used as markers.

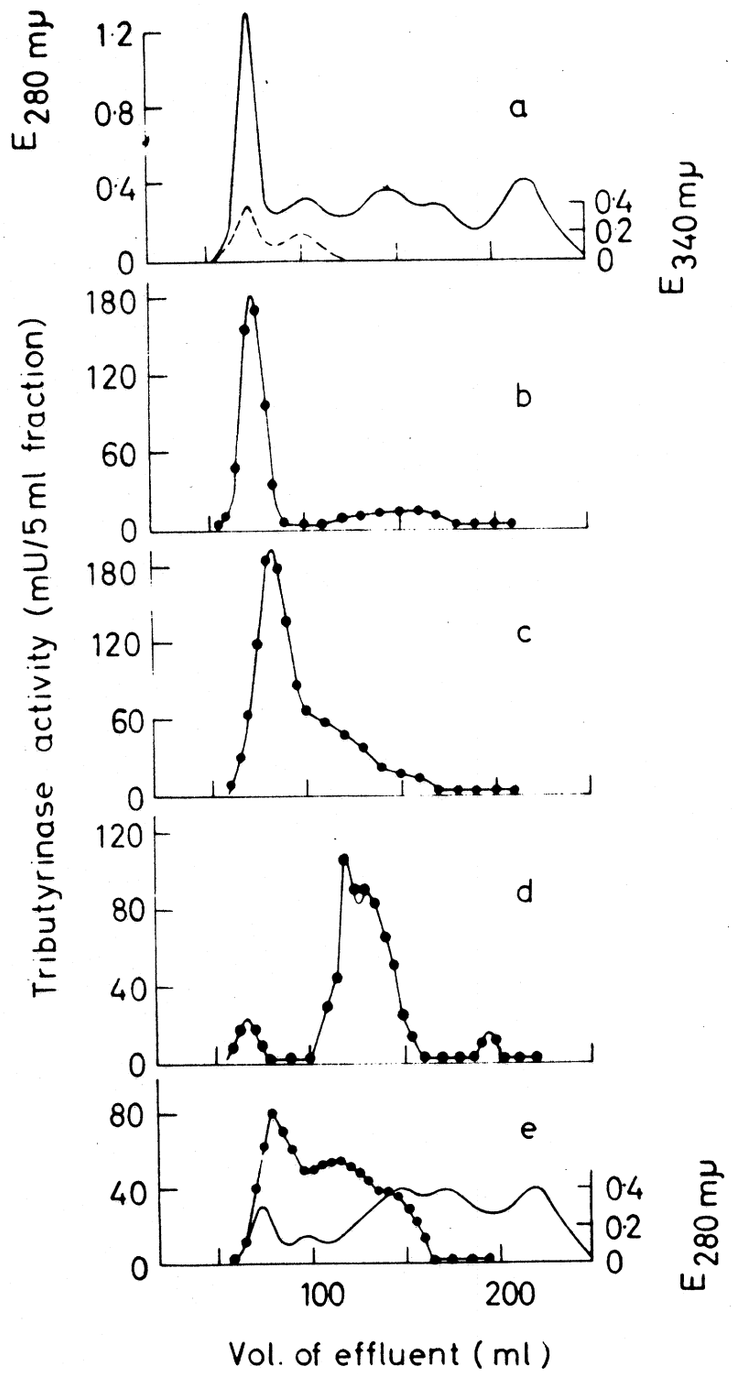


Fig. 5. Effect of NaCl concentration on the gel-filtration behaviour on Sephadex G-200 columns of the lipase (●——●) in two NaCl-supernatant preparations, obtained from skim milk as described in the text. One preparation is shown in (a)-(d) and the other in (e). Each run was performed with 4 ml. of either preparation. Elution diagrams for protein (———, extinction at 280 mμ) in each preparation are shown in (a) and (e). The presence of casein micelles and β-casein aggregates is shown in (a) by extinction at 340 mμ (---) measured at 25° (the explanation is given in the text). Columns were equilibrated with salt solutions as follows: (a) and (b), 0.05 M-NaCl containing 0.025 M $MgCl_2$; (c) and (e), 0.2 M-NaCl containing 0.025 M-$MgCl_2$; (d), 0.75 M-NaCl containing 0.025 M-$MgCl_2$

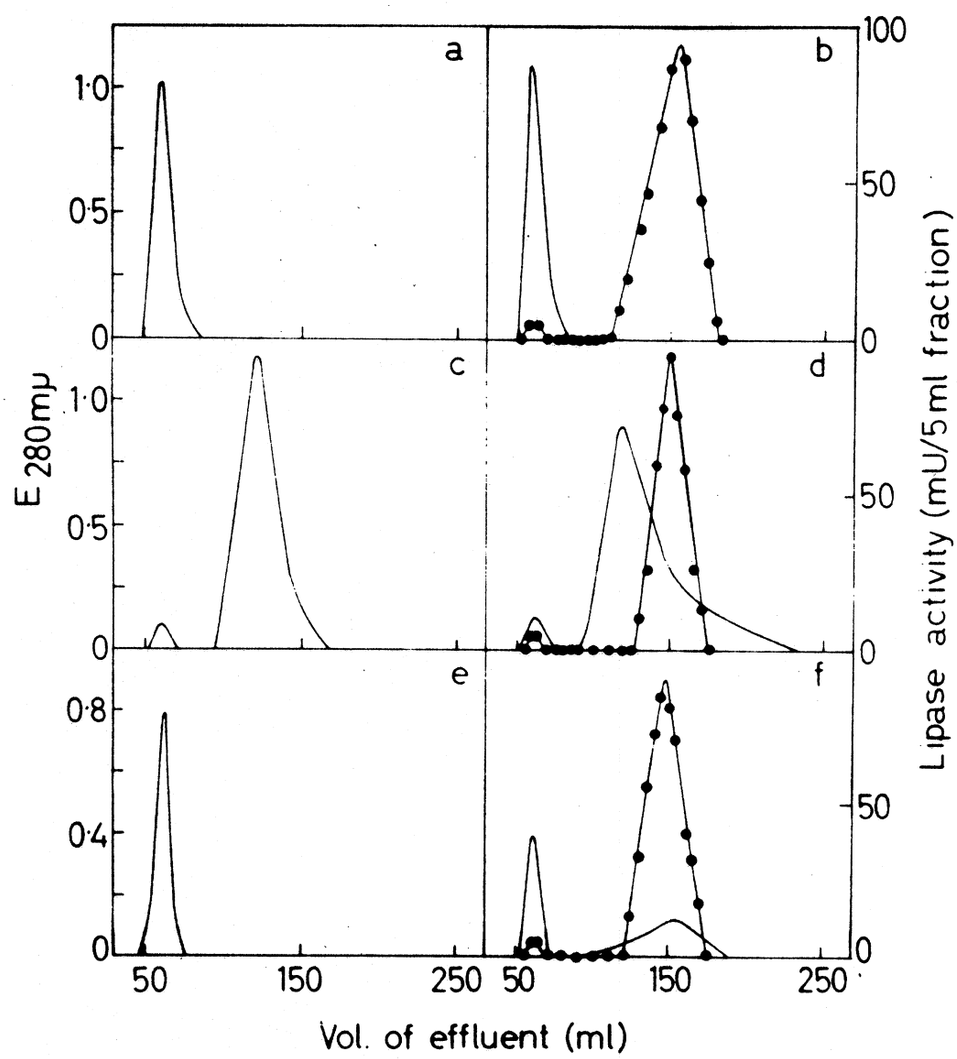


Fig. 7. Gel filtration on Sephadex G-200 columns of: sodium α-caseinate, 5 mg., (a) alone and (b) plus pancreatic lipase; sodium β-caseinate, 10 mg., (c) alone and (d) plus pancreatic lipase; sodium κ-caseinate, 7 mg., (e) alone and (f) plus pancreatic lipase. Details are given in the text. ——, Extinction at 280 mμ; •——•, lipase activity.

Effect of sample size and sodium chloride concentration on the gel-filtration behaviour of lipases in NaCl-supernatant (B). Changes in sample size and NaCl concentration in column eluents had much less effect on the gel-filtration behaviour of lipases in NaCl-supernatant (B) than they did on their behaviour in NaCl-supernatant (A). Increasing the sample volume of NaCl-supernatant (B) to 30 ml. in runs on a column equilibrated with 0.75 M-NaCl containing 0.025 M-$MgCl_2$ resulted in only small relative shifts of the higher molecular weight lipases towards the column void volume (Fig. 8 and Table 4).

In runs in 0.1 M-NaCl containing 0.025 M-$MgCl_2$, the sample volume was increased to 10 ml. before the lipases were eluted mainly at the column void volume.

Substrate specificity of lipases in NaCl-supernatant (B). The spread of the main lipase band in column effluents resulting from the use of relatively large sample volumes of NaCl-supernatant (B) caused little if any loss in peak definition and revealed the presence in it of five lipases. For substrate specificity studies, samples (10-30 ml.) of NaCl-supernatant (B) (containing 0.5-1.0 U of lipase activity/ml.) prepared from four different milks were submitted to gel filtration on columns equilibrated with 0.75 M-NaCl + 0.025 M-$MgCl_2$. Each effluent fraction was assayed within 2 hours of collection with tributyrin emulsion and either milk fat emulsion, triolein emulsion or triacetin solution as substrate. Percentage recoveries of activity were greater the larger the sample volumes.

Results obtained with preparations from one milk, which are similar to those obtained with two other milks, are shown in Fig. 8. No activity was eluted at the column void volume, and high buffering capacity in the fractions containing low molecular weight material precluded the estimation of lipase in this region. Each of the five enzymes which were detected (designated 1-5 in Fig.8) hydrolysed all four substrates, but generally at different rates. Enzymes 3, 4 and 5 hydrolysed tributyrin emulsion at faster rates than they hydrolysed milk fat emulsion, whereas 1 and 2 showed the opposite specificity (Fig. 8a). All five enzymes hydrolysed tributyrin emulsion faster than they hydrolysed triolein emulsion (Fig. 8b) but the preference of 1 and 5 for the former substrate was less pronounced than that of the other enzymes. All five hydrolysed triacetin in solution much more slowly than they hydrolysed any of the other three substrates (Fig. 8c).

(4) Interpretation of the gel filtration and other experiments on lipase-casein interaction.

Dissociation of the casein micelle-lipase complex. Comparison of the casein and lipase contents of high-speed supernatants prepared from salted and unsalted skim milk (Table 3) shows the separation of lipase from casein in the presence of sodium chloride. Dilution of milk reduces the size of the casein micelles (Nitschman, Helv. Chim. Acta, 32, 1259:1949) and both dilution and sodium chloride addition are believed to cause their partial dissolution (Hippel & Waugh, J. Amer. Chem. Soc. 77, 4311:1955; Zittle & Jasewicz J. Dairy Sci. 45, 703:1962), although a comparison of the data in Table 3 for supernatant (B) and NaCl-supernatant (B) indicates that the amount of sedimentable casein in skim milk is hardly altered by the presence of 0.75M-sodium chloride. At any rate, a concomitant release of lipase

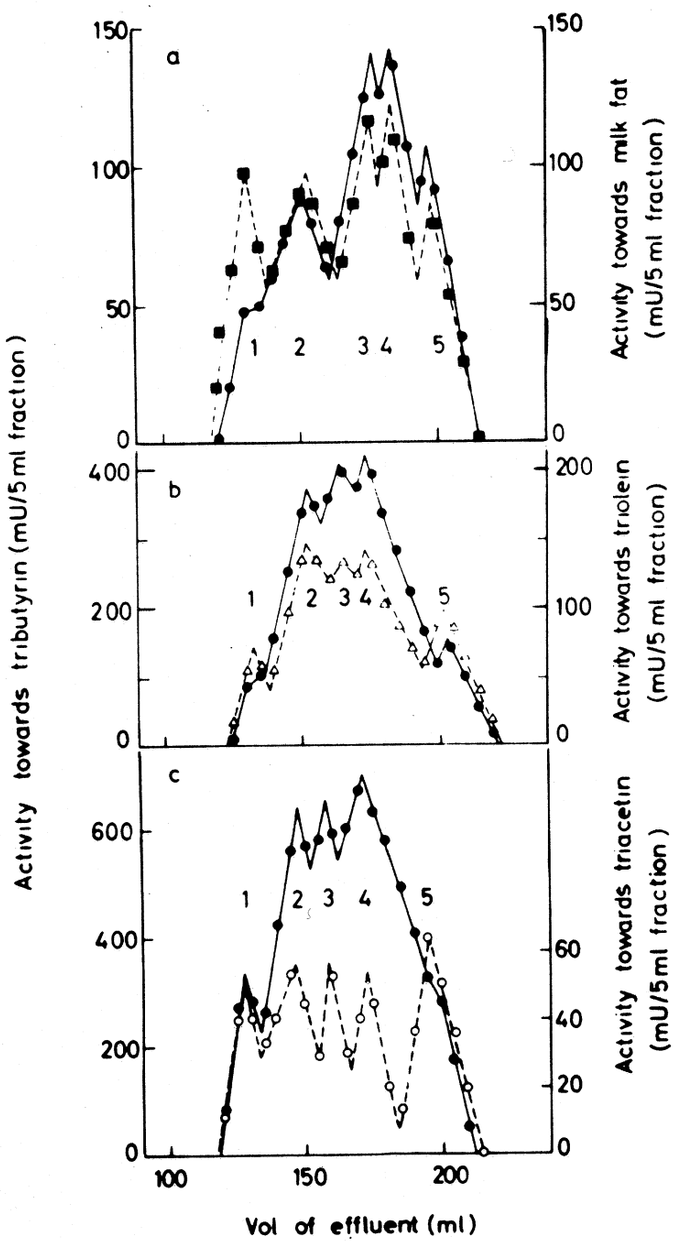


Fig. 8. Gel filtration elution diagrams(Sephadex G-200) of the lipases in NaCl-supernatant (B) (a, 10 ml.; b, 20 ml.; c, 30 ml.) prepared from skim milk as described in the text. For assays with tributyrin emulsion (●——●) or milk fat emulsion (■——■) as substrate, 1-2 ml. portions of the effluent fractions were used; for assays with triolein emulsion (Δ- - -Δ) or triacetin solution (o- - -o) as substrate, 3-3.5 ml. portions were used. The runs were performed on different columns, calibrated for molecular weight estimation (see Table 3 for results).

could well occur in either case. The extents to which dilution and sodium chloride addition increase the lipase activities of different milks might therefore be related to initial micelle size in the milks, the larger micelles more effectively masking the activity of the associated lipases.

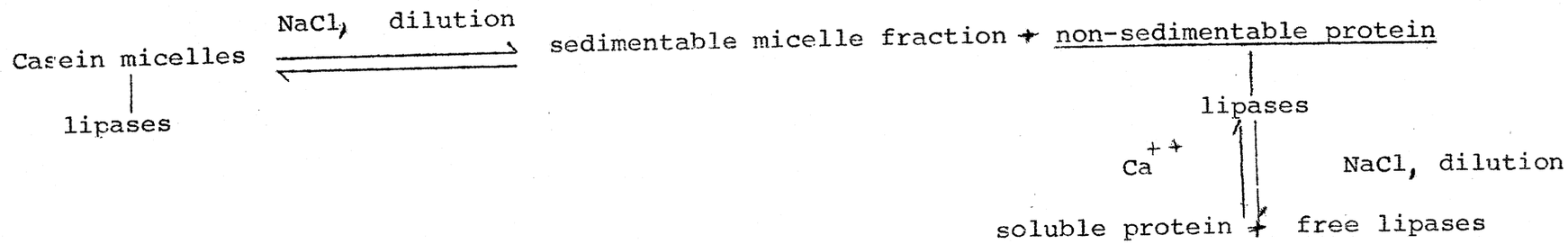
Noble & Waugh (J. Amer. Chem Soc. 87, 2236:1965) and Waugh & Noble (J. Amer. Chem. Soc., 87, 2246: 1965) suggest that some of the casein in micelles is in equilibrium with casein in solution, and if the lipases in non-sedimentable form in milk are still bound to casein (see below) they may be involved in the equilibrium. But if the micelle-lipase complexes dissociated only into non-sedimentable material, to some of which the lipasesare bound, sedimentation of the undissociated micelles will result in reassociation of the remaining material in an attempt to restore equilibrium. Even if the presence of sodium chloride alters the equilibrium concentrations of the components of the system, prolonged centrifugation of unsalted or salted skim milk will eventually give supernatant solutions with the same contents both of casein and of lipase. This, however, is not the case (Table 3). The observations are better explained if the micelle-lipase complexes are in equilibrium with a sedimentable micelle fraction plus a much smaller amount of non-sedimentable protein to which the lipases are still attached (Scheme 1). If the micelle-lipase complexes and the sedimentable micelle fraction sediment at equal rates, the equilibrium concentration of lipase should remain in the supernatant. However, if the micelle-lipase complexes sediment more rapidly than does the other material, the lipase concentration will be reduced. For example, calculation shows that if the latter material sediments 80% as rapidly as do the complexes, the final lipase concentration in the supernatant will be about 15% less than the equilibrium concentration. Neglecting any effect due to unequal sedimentation rates, our results indicate that in skim milk the complete micelles are associated with about 70% of the lipase, whereas in skim milk containing 0.75 M-sodium chloride only about 30% of the lipase is associated with micelles.

The non-sedimentable protein-lipase complex. The occurrence of lipases in bound form after the dissociation of micelle-lipase complexes is shown by the results of gel filtration experiments with various amounts of NaCl-supernatant (A) (Fig.4). Increases in sample size, with the attendant lessening in dilution during passage through the column, resulted in progressive decreases in the elution volumes of the lipases and decreases in the separations between them. The results suggested that in the experiment with an 18 ml. sample (Fig. 4a) the elution volume of the main lipase peak was characteristic of the state of the lipases in undiluted NaCl-supernatant (A). The interpretation is that the non-sedimentable protein-lipase complexes have molecular weights of roughly 350,000 and that dilution induces their dissociation to free lipases and soluble protein (Scheme 1). Changes in the lipase elution pattern are most evident when the diagrams for 4 ml. and 6 ml. samples are compared (Figs. 4e and 4c respectively), indicating that in 0.75 M-sodium chloride a small change in their concentration is sufficient to induce dissociation of the non-sedimentable protein-lipase complexes. Since the elution diagrams for 3 ml. and 4 ml. samples are similar (Figs. 4e and 4f respectively), the concentration change for dissociation presumably occurs very near the beginning of a column run when sufficiently small samples and a sufficiently high salt concentration are used. Dissociation evidently occurs less readily in sodium chloride concentrations lower than 0.75M, for in 0.2 M-sodium chloride

Table 4. Effect of sample size on the gel-filtration behaviour of lipases in NaCl-supernatant (B)

The corresponding elution diagrams are shown in Fig. 8. The apparent molecular weights are derived from the elution volumes of the tributyrinase peaks, which are numbered 1 to 5 in order of increasing elution volume in Fig. 8.

Elution diagram	Sample vol. (ml.)	Apparent mol. wts. of lipases 1	2	3	4	5	Recovery of tributyrinase activity (%)
Fig. 5a	10	198000	118000	80000	62000	45000	35
Fig. 5b	20	209000	128000	81300	63000	44000	53
Fig. 5c	30	257000	138000	89000	63000	46000	70



Scheme 1. Suggested model for the reversible casein micelle-lipase interaction. The model and relevant experimental observations are discussed in the text.

some of the lipase in 4 ml. of NaCl-supernatant (A) was eluted still as protein-lipase complex, and the remainder as free enzyme resulting from dissociation during various stages of the column runs (Figs. 5c 5e).

The effect of sample size on the gel-filtration behaviour of the five lipases detected in NaCl-supernatant (B) (Table 4) suggests that the affinity of the enzymes for complex formation with the non-sedimentable protein varies regularly with their molecular weights, the one with the lowest molecular weight having the least affinity.

Formation of casein micelle-lipase complexes. Complete reversal of Scheme 1, the conversion of free lipase to micelle-bound lipase, was observed when pancreatic lipase was added to NaCl-supernatant (A) and the mixture submitted to gel filtration on a column equilibrated at a low salt concentration (Fig. 6c). When saturation of the micellar lipase-binding capacity was achieved in an experiment with a relatively large amount of pancreatic lipase (Fig. 6d) the amount of lipase activity eluted with the micelle peak was about fifty times the amount of milk lipase activity usually found with micelles under similar conditions. Re-incorporation of non-sedimentable protein-milk lipase complexes into micelles occurred when the sodium chloride concentration in NaCl-supernatant (A) was lowered sufficiently, for when 4 ml of NaCl-supernatant (A) was run on a column equilibrated with 0.05 M-sodium chloride most of the lipase was eluted with the casein micelle peak (Fig. 6b).

Possible identity of the non-sedimentable lipase-binding protein. The protein is probably a natural micellar constituent, for lipase in milk is normally associated with the casein micelles. As pancreatic lipase still behaved as free enzyme during gel filtration at low salt concentration when mixed with an NaCl-supernatant (A) preparation containing very little casein (Fig. 6b), The lipase-binding protein appears to be a casein fraction. However, a comparison of the casein content of NaCl-supernatant (b) with that of supernatant (B) (Table 3) indicates that the amount of casein involved in lipase binding is very small, relative to the total amount of casein in milk.

Involvement of the hetergeneous α-casein fraction in lipase binding was suggested by its similarity to milk lipase in migration rate in continuous paper electrophoresis (Skean & Overcast J. Dairy Sci., 44, 8231 (1961). The possibility of the lipase-binding material being K-casein, which is part of the α-casein fraction, was proposed by Yaguchi, Tarassuk & Abe (J. Dairy Sci., 47, 1167:1964) following their observation that lipase activity was to some extent correlated with the amount of sialic acid in fractions from DEAE-cellulose chromatography of lipase-containing casein. However, K-casein may not be the only sialic acid-containing casein fraction (Cayen, Henneberry & Baker, J. Dairy Sci., 45, 706:1962) and we found that almost all the sialic acid was removed from skim milk in the preparation of NaCl-supernatant (B) (Table 3). Although the trace of sialic acid remaining in NaCl-supernatant (B) could be part of the lipase-binding protein, identification of the latter as K-casein is rendered uncertain. The possible identity of K-casein with milk lipase, as suggested by Yaguchi et al. loc. cit. certainly seems unlikely.

Some observations suggest that β-casein is the lipase-binding

protein. β-Casein aggregates in NaCl supernatant (A), detected by gel filtration experiments, were eluted in a position corresponding to a molecular weight of about 200,000 (Fig. 5a). Purified β-casein behaved similarly but α- and K-caseins were eluted from a Sephadex G-200 column at the void volume (Fig. 7). Thus simple stoicheiometric association of lipases with molecular weights in the range 45000-150000 (see below) with β-casein could result in the lipases being eluted from columns in the observed position of the non-sedimentable protein-lipase complex, whereas the same possibility does not exist for α- or K-casein. Saito & Hashimoto (Jap. J. Zootechnical Sci., 34, 393:1963) found similarities in the behaviour of milk lipase and β-casein during anion-exchange chromatography of preparations from milk, and Shahani & Chandan (Arch. Biochem. Biophys. 111, 257:1965) observed complex formation between clarifier slime lipase and β-casein, but other caseins were not tested in this respect. The model for casein micelle structure discussed by Noble & Waugh (loc. cit.) and Waugh & Noble (loc.cit.) has calcium a_s- and β-caseinates as core materials, which are not in exchange with the environment, and a coat of calcium a_s- and K-caseinates which are in exchange with similar components in solution. Since milk lipases associated with casein micelles are not fully active, lipase-binding to core material, with some impedance of substrate access to enzyme, seems likely. On the other hand, if lipase is involved in the equilibrium between micellar casein and casein in solution, as some of our results suggest, then lipase-binding to coat material is indicated. Clearly the identity of the non-sedimentable lipase-binding protein is uncertain. Perhaps lipase will associate with various casein fractions, as indicated by the polyacrylamide electrophoretic experiments of Gaffney & Harper (J. Dairy Sci., 48, 613:1965), depending on the conditions (see below). However, it has yet to be proved that the reported similarities in electrophoretic or chromatographic behaviour between casein fractions and milk lipases result from association between them, so the possibility remains that if caseins are not the only protein constituents of micelles, then lipase-binding to non-casein proteins occurs.

Possible nature of the lipase-protein binding. Gel filtration experiments show that wheat germ esterase (Fig. 6c) and other enzymes have little or no tendency to associate with the lipase-binding protein of milk under conditions in which lipases are readily bound. In view of the specific preference of lipases for attacking emulsified rather than soluble substrates the suggestion is that the attachment of lipase to an interface (cf. Benzonana & Desnuelle, Biochim. Biophys. Acta, 105, 121?1965) and its association with the lipase-binding protein in milk are similar phenomena. This association would not be expected to decrease the lipase activity as does the inclusion of lipase into casein micelles, and this is in accord with the observations recorded here.

Pancreatic lipase fails to associate with the soluble sodium α-, β- and K-caseinates in dilute sodium chloride solution (Fig. 7), but this does not necessarily exclude the caseins from the role of lipase-binding protein. The solubility properties of caseinates are such (Waugh, J. Phys. Chem., 65, 793:1961; Noble & Waugh, loc. cit.; Waugh & Noble, loc. cit.) that some of the calcium or calcium/sodium caseinates may be able to exist in forms, neither wholly in solution nor easily sedimentable at 80000g nor of very high molecular weight, which are suitable for attachment of lipase. Thus various caseins or even mixed caseins might be

involved in lipase binding, the identity of the casein being determined by the ionic content of the milieu. This could account for the conflicting evidence for the identity of the lipase-binding material.

Effect of casein micelle concentration on the formation of free lipase. Simple kinetics applied to Scheme 1 do not explain why the higher the casein micelle content of the NaCl-supernatant preparations, the less readily do the non-sedimentable protein-lipase complexes dissociate on gel filtration to give free lipases. The effect is shown in gel filtration experiments with different NaCl-supernatant (A) preparations (Figs. 5c, 5e) and by comparing the influence of sample size and ionic strength on the gel filtration behaviour of lipases in NaCl-supernatant (A) with their influence on those in NaCl-supernatant (B). A possible explanation follows from our suggestion that formation of the non-sedimentable protein-lipase complex depends on the calcium content of the preparation. No doubt the calcium in skim milk containing sodium chloride is present partly in the micelles and partly in the soluble phase, and exchange occurs between the two (Waugh loc. cit.). If calcium caseinate rather than sodium caseinate is preferentially sedimented in the micelles (cf. Noble & Waugh, loc. cit; Waugh & Noble, loc. cit.) the calcium sodium ratio in the supernatant will decrease as sedimentation proceeds. Weaker binding between non-sedimentable protein and lipases will result. The greater dissociation of non-sedimentable protein-lipase complexes in 0.75 M-sodium chloride as compared with 0.2 M-sodium chloride (Fig. 5d compared with Figs. 5c and 5e) also accords with a connection between binding strength and a cation ratio.

Multiplicity and molecular weights of milk lipases. Clearly, the uncertainty of molecular weight estimations based on the gel filtration behaviour of these enzymes is increased by their interaction with other proteins. Fortunately the experiments with NaCl-supernatant (A) (Fig. 4) indicate that when 3 ml. or 4 ml. samples were used, dissociation of the protein-lipase complex occurred very early in the column runs and therefore the interaction probably had little influence on the molecular weight estimations already published (Downey & Andrews, Biochem. J. 94, 642:1965).

When the protein-lipase interaction was used to improve the separation of the lipases, the presence of five enzymes instead of three was revealed (Fig. 8). Comparison of the elution diagrams and estimated molecular weights (Table 4) with those already published indicates that the enzymes recognized before are those designated 2, 3, and 4 in Fig. 8. Estimated molecular weights for the other two enzymes are 45000 for 5, since according to Table 4 little if any allowance for protein interaction seems necessary, and about 180,000 for 1, some allowance being made for protein interaction.

There is the possibility that the multiplicity of milk lipases arises from the association of one enzyme with casein or other proteins. It is suggested that the results of the substrate specificity studies, the observed dissociation of non-sedimentable protein-lipase complexes, and the arguments given previously (Downey & Andrews, loc. cit.) render this interpretation unlikely. The results of numerous other workers indicate that milk contains more than one lipase, but correlation between these results and ours remains to be established.

(5) Clarifier slime lipase.

Whether or not the lipase of molecular weight 7,000 isolated from milk clarifier slime and studied by Chandan & Shahani (J. Dairy Sci., 46, 275, 403:1963), Chandan, Shahani, Hill & Scholz (Enzymologia, 26, 87:1963) and Shahani & Chandan (Arch. Biochem. Biophys., 111, 257:1965) is a true milk lipase is uncertain. It might be represented in gel filtration elution diagrams of NaCl-supernatant (A) preparations by a small, variable peak of lipase activity in the region of low molecular weight material (cf. Downey & Andrews, loc. cit.). However, the characterization of the enzyme of this peak in terms of substrate specificity was not possible because when larger sample volumes were used because the buffer action in this region rendered the titrimetric assay uncertain, neither could the enzyme be detected in extracts of clarifier slime. Attempts were made to isolate lipase from four samples of clarifier slime by the procedure of Chandan & Shahani (J. Dairy Sci., 46, 275:1963). Lipase activities in aqueous extracts of the slime ranged from 0.3 to 2.1 U/ml., but the activities of preparations obtained after acetone and ammonium sulphate fractionation of the extracts were insufficient for further experiments. Gel filtration of the slime extracts was impeded by their very high viscosities in the presence of 0.75 M-NaCl + 0.025 M-$MgCl_2$. Lipase recovered in low yield (10-20%) from some runs with this eluent emerged from the column only with the casein micelle peak. Therefore, during fractionation the material behaved unlike that described by Chandan & Shahani.

The 22% recovery of purified lipase from clarifier slime reported by Chandan & Shahani (J. Dairy Sci., 46, 275:1963) indicates that the enzyme formed a major part of the slime lipase. On the other hand, as the specific activity of lipase in clarifier slime is about three times that in whole milk on a protein basis (Nelson & Jezeski J. Dairy Sci., 38, 479: 1955), calculations based on data given by Chandan & Shahani (loc. cit.) together with a figure of 30 g./100 ml. as the protein content of clarifier slime (Hökl & Štěpánek (Hygeine der Milch und Milcherzeugnisse, p. 177: veb Gustan Fischer, Jena, 1965) show that the purified slime lipase equals only about 0.1% of the lipase activity of the original milk, so the possibility that the slime lipase represents the overall lipase content of milk is rendered unlikely. An alternative origin for the purified slime lipase is indicated by the results of Gaffney & Harper (J. Dairy Sci., 48, 613:1965), who have found lipase in somatic cells obtained from separator slime.

V NON-ENZYMIC ESTERASE ACTIVITY IN MILK

Esterase activity is often determined by measuring the catalysed hydrolysis of aryl esters. However, in an attempt to separate and characterize the reported cow's-milk esterases (Forster, Bendixen & Montgomery, J. Dairy Sci., 42, 1903:1959; Forster, Montgomery & Montoure, J. Dairy Sci., 44, 1420:1961) by their gel-filtration behaviour it was found that only a small part of the esterase activity of milk towards p-nitrophenyl acetate can be attributed to enzymes. The hydrolysis of p-nitrophenyl acetate by serum albumin is well documented, and Hartley & Kilby (Biochem. J., 56, 288:1954) have suggested that the property is common to other non-enzymic proteins.

Methods. Hydrolysis of p-nitrophenyl acetate was measured by

spectrophotometric estimation of the liberated p-nitrophenyl at 410 mμ with 1 cm. cuvettes and total volumes of 3 ml. at 25°. Assay mixtures contained 200 μmoles of tris hydrochloride buffer, pH 7.0, 15 μmoles of p-nitrophenyl acetate (added as a solution in 0.4 ml. of methanol) and 100 μmoles of NaCl. Extinction in cuvettes containing no esterase increased by 0.03/min., corresponding to the spontaneous hydrolysis in the assay mixture of 12 mμ moles of substrate/min. Whenever practicable, sufficient esterase solution (e.g. 0.1 ml. of skim milk or 1-2 ml. of column effluent) to double this hydrolysis rate was used in the assays, and the results were appropriately corrected for the blank. Extinction increased linearly for at least 30 min.. Gel filtration was carried out on a Sephadex G-200 column as described above.

Friesian cows yielded skim milk with esterase activities/ml. sufficient to hydrolyse 80-160 mμ moles of p-nitrophenyl acetate/min. Manometric measurements of acid liberation from phenyl acetate by Forster, Montgomery & Montoure (loc. cit.) gave the range 60-600 mμmoles hydrolysed/ml. of skim milk/min. at 37°. The addition of NaCl to the skim milks to 0.75M final concentration and centrifugation at 80,000 g for 1 hr. to remove casein gave opalescent solutions (NaCl-supernatant (Λ)) containing about 60% of the skim-milk esterase.

Several NaCl-supernatants were submitted to gel filtration and the effluent fractions assayed for protein (extinction at 280mμ) and for esterase activity. Results for a typical preparation are given in Fig. 9a. Columns equilibrated at high or at low salt concentrations gave similar results. The peaks with elution volumes of 70, 105, 170, 195 and 237 ml. probably represent casein micelles, milk immune globulins, β-lactoglubulin, α-lactalbumin and low molecular weight material respectively. Esterase activity (90% recovery) was found in all the fractions containing protein or low molecular weight material. Although the ratio between protein concentration and esterase activity differed from one fraction to another, some similarity between the protein and esterase elution diagrams is evident.

Esterase activity of purified milk proteins. Purified milk proteins catalysed the hydrolysis of p-nitrophenyl acetate as follows (expressed as mμ moles of substrate hydrolysed/mg. of protein/min.): α-casein, 3.1; β-casein, 3.0; k-casein, 2.8; β-lactoglobulin A, 4.5; α-lactalbumin, 3.7. The esterase activities of bovine serum albumin and γ-globulins, on the same basis, were 14.3 and 7.5 respectively. When these proteins were subjected separately to gel filtration on the Sephadex G-200 column equilibrated with 0.1 M-NaCl, the esterase activities were recovered quantitatively in peaks corresponding exactly to those of the proteins themselves. Results with α-casein, β-lactoglobulin A and serum albumin are shown in Figs. 9b-9d respectively.

Heating at 60° or 80° for 2 min. destroyed only 10-20% of the esterase activities of skim milks and of NaCl-supernatants prepared from them, and heating for another 30 min. effected no further change. When the fractions from a gel filtration run with NaCl-supernatant were similarly heated, the overall esterase loss (18%) occurred mainly in fractions from the region of whey proteins (Fig.9e), indicating the presence therein of small amounts of enzyme. Solutions of the purified milk and serum proteins (3mg./ml. in 0.1 M-NaCl) lost no esterase activity when heated at 60° for 30 min; heating at 80° precipitated some of the protein.

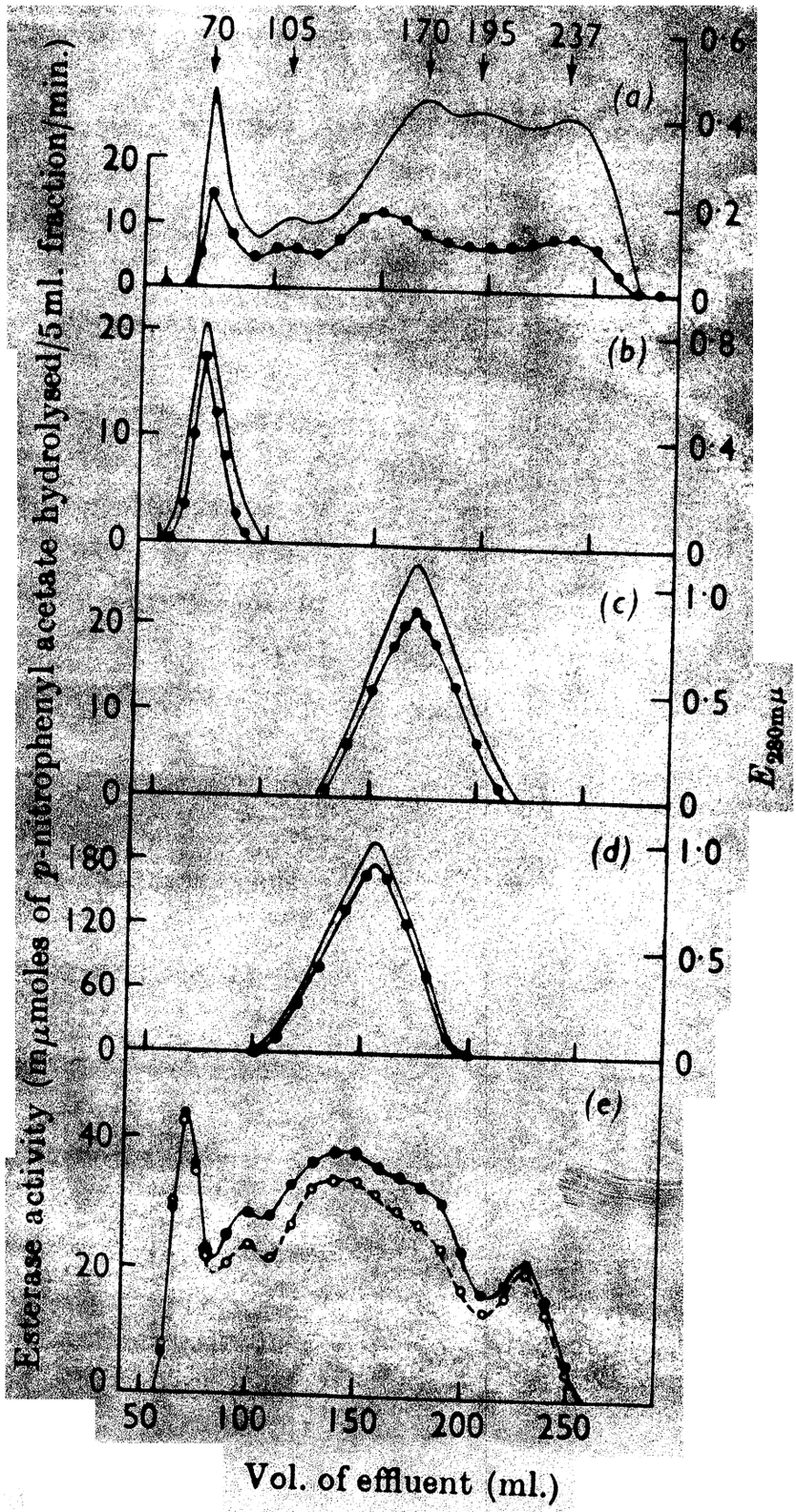


Fig. 9 Gel filtration of NaCl-supernatants from cow's milk and purified proteins on Sephadex G-200 columns. (*a*) NaCl-supernatant (4ml.); (*b*) α-casein (20mg.); (*c*) β-lactoglobulin A (50mg.); (*d*) bovine serum albumin (100mg.); (*e*) NaCl-supernatant (10ml.). Samples for (*a*) and (*e*) were prepared from different milks. ——, Extinction at 280mμ; ●—●, esterase activity of unconcentrated effluent fractions; ○---○, esterase activity of concentrated effluent fractions.

The results indicate that of the low esterase activity of cow's milk, as measured by hydrolysis of p-nitrophenyl acetate, about 70% is due to non-enzymic proteins, about 10% is due to catalytic activity exhibited by low molecular weight material, and only about 20% is due to enzymes with conventional heat-lability. Calculations based on the specific esterase activities given above for purified proteins, the data given by Jenness & Patton (in Principles of Dairy Chemistry, p. 2; 1959) on the protein composition of cow's milk, and the assumption that caseins in micellar form have the same esterase activities as purified caseins in solution, give a value of 115 mμmoles of p-nitrophenyl acetate hydrolysed by the non-enzymic proteins/ml. of milk/min. Additional activity due to enzymes and low molecular weight material gives a total of about 160 mμmoles of substrate hydrolysed/ml. of milk/min., in good agreement with observed values. As the calculated activity due to casein is about half this total, the predicted effect of sedimentation of casein also accords with observation. Clearly, some caution is necessary before attributing low esterase activities in crude preparations to true enzymes.